PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research

ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 39(11):915-922, November 2019
DOI: 10.1590/1678-5150-PVB-6158

Original Article
Small Animal Diseases

Genotyping of *Malassezia pachydermatis* disclosed genetic variation in isolates from dogs in Colombia[1]

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ABSTRACT.- Celis-Ramírez A.M., Guevara-Suarez M., Galvis-Marín J.C., Rodríguez-Bocanegra M.X., Castañeda-Salazar R., Linares-Linares M.Y., Triana-Sierra S.H. & Pulido-Villamarín A.P. 2019. **Genotyping of *Malassezia pachydermatis* disclosed genetic variation in isolates from dogs in Colombia**. *Pesquisa Veterinária Brasileira 39(11):915-922.* Unidad de Investigaciones Agropecuarias, Departamento de Microbiología, Facultad de Ciencias, Pontificia Universidad Javeriana, Carrera 7 43-82, Ed. 52, Of. 608, Bogotá, Colombia. Departamento de Ciencias Biológicas, Universidad de Los Andes, Carrera 1 18A-10, Bloque J, Of. J-214, Bogotá, Colombia. E-mails: adriana.pulido@javeriana.edu.co, acelis@uniandes.edu.co

Malassezia pachydermatis is a lipophilic and lipid-dependent yeast mostly isolated from animals' skin; hence, it is regarded as a zoophilic species causing otitis externa in dogs. Aspects associated with its epidemiology and pathogenicity is a matter of interest. This study aimed to conduct a molecular characterization of 43 isolates of *M. pachydermatis* obtained from dogs with otitis externa. For this purpose, the 5.8S internal transcribed spacer 2 (ITS2) and D1/D2 26S rRNA regions were amplified, sequenced and analyzed using restriction fragment length polymorphism (RFLP) with *Alu*I, *Cfo*I, and *BstF5*I endonucleases. Phylogenetic analyses revealed that these isolates grouped with the sequence types I, IV and V, previously proposed for *M. pachydermatis*. Interestingly, we found a new polymorphic RFLP pattern using *BstF5*I, these isolates were associated with the sequence types IV and V, nevertheless an association between polymorphic RFLP patterns, and fosfolipase activity or canine population data was not observed. These findings underline the genetic diversity of *M. pachydermatis* and provide new insights about the epidemiology of this species in the analyzed population.

INDEX TERMS: Genotype, *Malassezia pachydermatis*, genetic variation, isolates, dogs, Colombia, gene, otitis, PCR-RFLP, sequence types.

RESUMO.- [Genotipagem de *Malassezia pachydermatis* revelou variação genética em isolados de cães na Colômbia]. *Malassezia pachydermatis* é uma levedura lipofílica e dependente de lipídios, principalmente da pele de animais. Sendo, por essa razão, considerada uma espécie zoofílica e causadora de otite externa em cães. Neste sentido, aspectos associados à sua epidemiologia e patogenicidade constituem um tema de interesse científico. O objetivo deste estudo foi realizar a caracterização molecular de 43 isolados de *M. pachydermatis* obtidos a partir de cães com otite externa. Para esta propósito, foram amplificadas, sequenciadas e analisadas com enzimas de restrição as regiões do gene 5.8S, do espaçador interno transcrito 2 (ITS2) e D1/D2 do 26S do rRNA pelo método RFLP, com as endonucleases *Alu*I, *Cf*OI e *BstF5*I. Análises filogenéticas revelaram que os isolados se agruparam com as sequências tipo I, IV e V de *M. pachydermatis* como já descrito anteriormente. De maneira interessante, se observou um novo RFLP polimórfico utilizando *BstF5*I. Os isolados que mostraram esse padrão foram associados com os padrões IV e V. No entanto, não foi observada associação entre padrões

[1] Received on April 24, 2019.
Accepted of publication on May 7, 2019.

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polimórficos de RFLP e atividade de fosfolipase ou dados da população canina. Estes resultados demonstram a diversidade genética de *M. pachydermatis* e fornecem novas perspectivas sobre a epidemiologia destas espécies na população analisada.

TERMOS DE INDEXAÇÃO: Genotipagem, *Malassezia pachydermatis*, variação genética, isolados, cães, Colômbia, genes, otite, PCR-RFLP, tipos de sequência.

INTRODUCTION

Malassezia genus comprises 18 species (Triana et al. 2015, Wu et al. 2015, Cabañes et al. 2016, Honnavar et al. 2016, Lorch et al. 2018), which are considered as the dominant species within animal and human skin mycobiota, characterized by being lipophilic and lipid dependent. Nevertheless, these commensal yeasts have also been related to several dermatological diseases (Sugita et al. 2010, Harada et al. 2015, Wu et al. 2015, Prohic et al. 2016).

Malassezia pachydermatis is one of the most common species within this genus, which is commonly isolated from mammals and birds. In dogs, it is frequently found in the external ear canal; moreover, this species may cause otitis externa and dermatitis (chronic, localized or generalized, external lesions) (Crespo et al. 2002, Bond et al. 2010, Pulido et al. 2010, Pulido-Villamarín et al. 2015). Furthermore, *M. pachydermatis* has been considered to be potentially zoonotic, mainly associated to fungaemia (Gueho et al. 1987, Morris et al. 2005, Bond et al. 2010, Seyedmousavi et al. 2015). Given the fact that a possible association of genotypes with specific hosts has been discerned, it can be suggested that *M. pachydermatis* has adapted to their ecological niche and could be related to the establishment of the disease (Puig et al. 2016). In addition, several studies have linked the pathogenicity of this species with phospholipase activity, biofilm production and immune response (Cafarchia et al. 2008, Buommino et al. 2016, Hurtado-Suárez et al. 2016).

Several studies have reported the isolation of *M. pachydermatis* from external ear canal samples of both ill and healthy canines (Bond et al. 1995, Duarte et al. 2001, Masuda et al. 2000, Castellá et al. 2005, Girão et al. 2006, Brito et al. 2007, Bond et al. 2010). In South America, only some reports regarding this matter has been conducted. In Brazil studies including canines and other domestic and wild animals reported the prevalence of *M. pachydermatis;* however, the identification of these isolates were mainly achieved using conventional methods (Duarte et al. 2001, Girão et al. 2006, Brito et al. 2007). Nevertheless, molecular biology techniques, such as restriction fragment length polymorphism (RFLP) and nucleic acid sequencing have become common place procedures. Molecular methods for species identification employ ribosomal DNA genes (rDNA) as diagnostic markers, including the 26S (D1D2-Fw/D1D2) and 5.8S (ITS3/ITS4) subunits (Mirhendi et al. 2005, Gaitanis et al. 2006, Ribeiro do Prado et al. 2007, Cafarchia et al. 2011a, Kim et al. 2015), this genotyping has allowed to identify high intra-species variability in *M. pachydermatis* with the presence of several sequence types (Guillot et al. 1997, Cafarchia et al. 2007, 2008, 2011b, Puig et al. 2016). These assessments have improved the characterization of these yeast populations to the better knowledge of their epidemiology.

PCR-RFLP is one of the most reliable techniques, usually trough the restriction enzyme digestion of LSU rDNA and ITS regions (Boekhout et al. 1998, Aizawa et al. 1999, Theelen et al. 2001, Gaitanis et al.2002, Celis & Cepero de García 2005, Mirhendi et al. 2005, González et al. 2009, Sugita et al. 2010, Amado et al. 2013). Nevertheless, other methods such as multi-locus phylogenetic analyses have evinced to be a useful tool to distinguish closely related species. Recently, these analyses showed that *M. pachydermatis* has a high intra-species variability due to the presence of several sequence types, at least five (I-V) for LSU and eleven (I-XI) for ITS (Puig et al. 2016). Moreover, the combination of LSU, ITS, CHS2 and β-tubulin gene sequences revealed the presence of fifteen genotypes clustered in two different clades; one clade mainly comprises isolates from dogs, while the other included isolates from other domestic animals such as cats (Puig et al. 2016).

To date, molecular characterization of *M. pachydermatis* isolates from animals in Colombia has not been reported. Therefore, the aim of the present study was to characterize at molecular level a set of isolates of this species obtained from dogs with otitis externa. This study can provide new insights on the genotypes of *M. pachydermatis* present in canine otitis.

MATERIALS AND METHODS

***Malassezia* isolates and growth conditions.** A total of 43 isolates of *M. pachydermatis* from dogs with clinical record of otitis externa from a previous study (Pulido et al. 2010) were included. The isolates were phenotypically characterized, including the evaluation of phospholipase activity (Pulido-Villamarín et al. 2015), preserved in 10% skin milk at -70 °C according to the methods for preservation and storage of *Malassezia* species (Crespo et al. 2000), and deposited at the microorganism collection from the "Unidad de Investigaciones Agropecuarias" (UNIDIA, Unit of Agricultural Research) of the "Pontificia Universidad Javeriana". In addition, *Malassezia* strains: *M. furfur* CBS 7019, *M. pachydermatis* CBS 1879, *M. sympodialis* CBS 7222 and *M. slooffiae* CBS 7956, obtained from the Fungal Biodiversity Center (Westerdijk Institute, Utrecht, The Netherlands), were used as phenotypic and genotypic controls. All strains were recovered using modified Dixon agar (mDixon agar; 36g L^{-1} Mycosel agar - BD, Franklin Lakes/NJ, USA -, 20g L^{-1} Ox bile, 36g L^{-1} malt extract - Oxoid, Basingstoke, UK -, 2mL L^{-1} glycerol, 2mL L^{-1} oleic acid, and 10 mL L^{-1} Tween 40), incubating for 4 to 5 days at 33°C (Mirhendi et al. 2005).

Molecular characterization. Total DNA was extracted using the fungal and yeast genomic DNA kit (Norgen Biotek, Thorold, ON, Canada), according to the manufacturer's instructions, and then treated with RNase (Sigma, St Louis/MO, USA) at a final concentration of 2mg/mL per sample at 37°C for 4 hours. The DNA samples were stored at -20°C until further use.

Two nuclear DNA targets were amplified by PCR and sequenced using primers previously reported (González et al. 2009): ITS3/ITS4 for the 5.8s rDNA-ITS2 region, and D1D2-Fw/D1D2 for 26S rDNA region (Table 1). Polymerase chain reaction amplification was carried out in a final volume of 50µL, where each reaction contained 45µL

Table 1. Forward and reverse primer sequences employed to amplify regions 5.8S rDNA-ITS2 and 26S rDNA

	Primer	Forward/reverse
5.8S rDNA-ITS2 region	ITS3	5'-GCATCGATGAAGAACGCAGC-3'
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'
26S rDNA region (D1D2)	D1D2-Fw	5'-TAACAAGGATTCCCCTAGTA-3'
	D1D2-Rv	5'-ATTACGCCAGCATCCTAAG-3'

Reference: Mirhendi et al. (2005).

Table 2. Origin of *the Malassezia pachydermatis* isolates included in the present study

Sequence type	Isolate no.	Breed	GenBank accession numbers	
			D1/D2	ITS
I	11	Labrador	KU757185	LT962403
I	27	Labrador	KU757188	LT962406
I	35	Labrador	KU757189	LT962407
I	43	Labrador	KU757194	LT962411
I	58	Labrador	KU757199	LT962415
I	80	Labrador	KU757203	-
I	100	Labrador	KU757207	LT962422
I	103	Labrador	KU757209	LT962424
I	104	Labrador	KU757210	LT962425
I	109	Labrador	KU757212	LT962427
I	128	Labrador	KU757220	LT962432
I	129	Labrador	KU757221	LT962433
I	131	Labrador	KU757222	LT962434
I	134	Labrador	KU757223	LT962435
I	135	Labrador	KU757224	LT962436
I	141	Labrador	KU757226	LT962438
I	149	Labrador	KU757231	LT962440
I	153	Labrador	KU757232	LT962441
IV	18	Labrador	KU757187	LT962405
IV	40	Labrador	KU757193	LT962410
IV	65	Labrador	KU757201	LT962417
IV	70	Golden Retriever	KU757202	LT962418
IV	83	Labrador	KU757205	LT962420
IV	111	Golden Retriever	KU757213	LT962428
IV	115	Stray dog	KU757214	LT962429
V	15	Labrador	KU757186	LT962404
V	36	Beagle	KU757190	LT962408
V	38	Golden Retriever	KU757191	LT962409
V	39	Golden Retriever	KU757192	-
V	47	Stray dog	KU757195	LT962412
V	54	Golden Retriever	KU757197	-
V	55	Beagle	KU757198	LT962414
V	59	Golden Retriever	KU757200	LT962416
V	81	Labrador	KU757204	LT962419
V	88	Golden Retriever	KU757206	LT962421
V	101	Stray dog	KU757208	LT962423
V	107	French Poodle	KU757211	LT962426
V	116	Labrador	KU757215	LT962430
V	125	French Poodle	KU757219	LT962431
V	140	Labrador	KU757225	LT962437
V	144	Golden Retriever	KU757228	LT962439
V	156	Beagle	KU757233	LT962442
V	157	Beagle	KU757234	LT962443

PCR SuperMix (1.1X) (Thermo Scientific, St Peters/MO, USA), 1µL of each primer (10pmol/µL), and 3µL of genomic DNA (20ng/mL). A first analysis with 26S rDNA and ITS sequences using the nucleotide BLAST tool[6] was carried out to confirm the preliminary phenotypic identification. The amplification protocol consisted of an initial denaturation step for 5min at 95°C and 30 cycles consisting of 95°C for 1min, 55°C for 1 min, and 72°C for 1min, followed by a final 5min extension step at 72°C.

The restriction enzyme digestion for the amplicon of 5.8S region was done with *Alu*I (Thermo Scientific, St Peters/MO, USA) and for 26S region with *Cfo*I (Promega, Madison/WI, USA) and *BstF5*I (Thermo Scientific, St Peters/MO, USA), enzyme reactions were performed as previously reported (Mirhendi et al. 2005). The products were visualized in 1.8% (p/v) agarose gel electrophoresis using a Gel Doc System (Bio-Rad, Hercules/CA, USA).

Single-band PCR products were purified and sequenced using an ABI3730xl DNA analyzer (Applied Biosystems, Foster City/CA, USA). Consensus sequences were assembled using Geneious software v.4.8.5[7]. DNA sequences determined in this study have been deposited in GenBank, the accession numbers are reported in Table 2.

Phylogenetic reconstructions. In order to assess the intraspecific variability of our isolates, we performed phylogenetic analyses using

[6] Basic Local Alignment Search Tool. Available at <https://blast.ncbi.nlm.nih.gov/Blast.cgi>

[7] Geneious Bioinformatics Software for Sequence Data Analysis. Available at <http://www.geneious.com>

the sequences data of D1/D2 and ITS and complemented them with sequence types reported for *M. pachydermatis* retrieved from GenBank database (Puig et al. 2016). Additionally, sequences of type strains of *Malassezia* species were included. For phylogenetic analyses, sequences of the individual loci were aligned using ClustalW algorithm (Thompson et al. 1994) in MEGA v. 7.0 (Kumar et al. 2016), refined with MUSCLE (Edgar 2004), and manually adjusted using the same software platform. Phylogenetic reconstructions were made with the individual loci using maximum-likelihood (ML) in MEGA v. 7.0 and Bayesian inference (BI) under MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001). The best substitution model for all matrices was estimated using JModelTest v. 2.1 (Darriba et al. 2012). For ML analyses, nearest-neighbor interchange was used as the heuristic method for tree inference. Support for internal branches was assessed by 1,000 ML bootstrapped pseudoreplicates of data. The phylogenetic reconstruction by BI was performed using 1,000,000 Markov chain Monte Carlo (MCMC) generations, with two runs (one cold chain and three heated chains), and samples were stored every 1,000 generations. The 50% majority-rule consensus tree and posterior probability (pp) values were calculated after discarding the first 25% of the samples as burn-in. The resulting trees were plotted using FigTree v. 1.4.2[8], and edited in Adobe Illustrator CS6. Bootstrap support (bs) and pp values were labelled on nodes of the three. Values of ≥70bs and less than 0.95pp were considered significant.

***In silico* restriction.** To confirm the restriction pattern for all the sequences obtained from 26S rDNA region with the enzyme *BstF5*I, *in silico* digestions were performed using the platform Webcutter 2.0[9].

RESULTS

For all isolates amplification of the 5.8S rDNA-ITS2 region resulted in a band of ~500bp, and a slightly larger band, ~550bp was obtained for the 26S rDNA region. An analysis with 26S rDNA and ITS sequences using the nucleotide BLAST tool allows us to confirm that all canine isolates were *Malassezia pachydermatis.* The restriction analysis obtained by PCR-RFLP agreed with what is reported for the identification of *M. pachydermatis,* using the 5.8S rDNA-ITS2 region with *Alu*I, and for 26S rDNA region restriction analysis using *Cfo*I. However, the digestion of 26S rDNA region with *BstF5*I revealed two different restriction patterns. The first pattern was found in 18 out of 43 isolates (Fig.1, lanes 2-4) as well as in the reference strain *M. pachydermatis* CBS 1879, this pattern consisted of two bands of ~450 and 60bp; the second pattern was observed in the remaining isolates (n=25), this consisted of four bands ~450, 300, 180, and 60bp (Fig.1, lanes 5-11). To confirm the new restriction pattern for *BstF5*I, we performed an *in silico* restriction, where we observed the results matched with an additional cutting site within the alignment of the 26S rDNA region.

Phylogenetic analyses performed to identify sequence types of *M. pachydermatis* based on 26S rDNA are shown in Figure 2. The final alignment with this region was 496bp long, and the model selected was Kimura 2-parameter (K2) with gamma-distributed rates (G). This analysis showed that all canine isolates formed a major clade (97% bs/1pp) with ex-type strain of *M. pachydermatis*. Additionally, these isolates

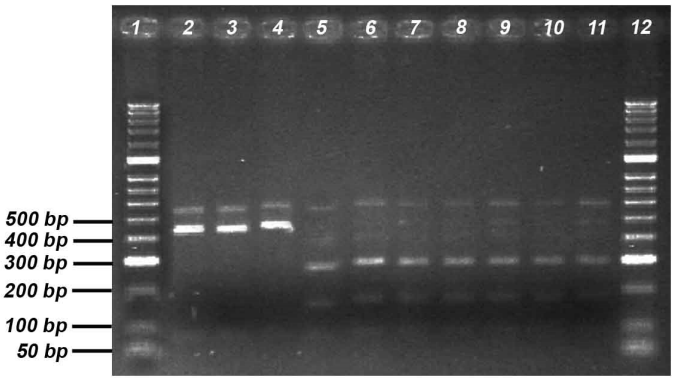

Fig.1. *Malassezia pachydermatis* 26S rDNA region amplicon digestion with *BstF5*I. Molecular-weight size marker (lane 1), *M. pachydermatis* CBS 1879 (lane 2), 26S rDNA region two pattern band (~450, 60bp) with *BstF5*I (lane 3-4), 26S rDNA region four pattern band (~450, 300, 180, 60bp) with *BstF5*I (lane 5-11), molecular-weight size marker (lane 12).

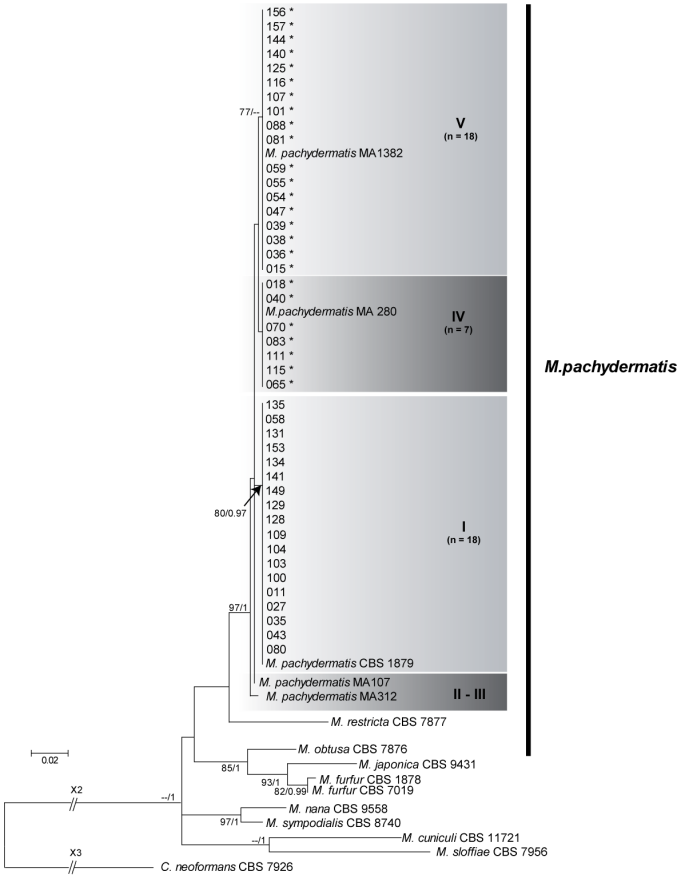

Fig.2. Phylogenetic diversity of isolates of *Malassezia pachydermatis* obtained from dogs in this study. Phylogenetic tree using maximum-likelihood (ML) and Bayesian inference (BI), tree inferred from 26S rDNA sequence data. Support values are above branches, and represent bootstrap values >70% for ML/posterior probabilities >0.95 for BI. The tree is rooted to *Cryptococcus neoformans* CBS 7926. Isolates with the four-banded pattern after digestion with *BstF5I* (*). The sequences types proposed by Puig et al. (2016) for 26S rDNA region are shown in dark boxes.

[8] Figtree Software, Molecular Evolution, Phylogenetics and Epidemiology. Available at <http://tree.bio.ed.ac.uk/software/figtree>

[9] WebCutter 2.0, A Restriction Mapping Tool. Available at <http://heimanlab.com/cut2.html>

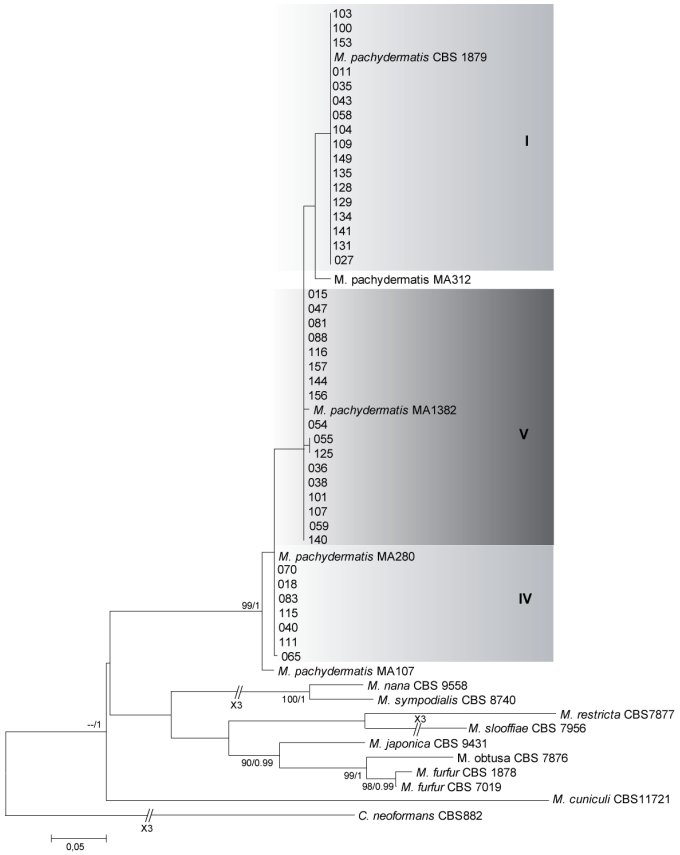

Fig.3. Phylogenetic diversity of isolates of *Malassezia pachydermatis* obtained from dogs in this study. Phylogenetic tree using maximum-likelihood (ML) and Bayesian inference (BI), tree inferred from 5.8S rDNA-ITS2 region. Support values are above branches, and represent bootstrap values >70% for ML/posterior probabilities >0.95 for BI. The tree is rooted to *Cryptococcus neoformans* CBS 882. The sequence types proposed by Puig et al. (2016) are shown in dark boxes.

were distributed among three sequence types, I (n=18), IV (n=7) and V (n=18), which have been previously reported. The analysis of the ITS sequences (Fig.3) showed similar topology to phylogenetic tree of 26S rDNA region. However, not all clades had a robust phylogenetic support.

Interestingly, we observed that two-banded *BstF5*I restriction pattern isolates grouped with the sequence type I, while the isolates with the four-banded pattern grouped with the sequence types IV and V (Fig.2). We did not find any association between the *BstF5*I restriction patterns for 26S rDNA and the phospholipase activity data (previously determined) nor with the breed, age, gender or ear type of the dogs (Pulido et al. 2010, Pulido-Villamarín et al. 2015, Hurtado-Suárez et al. 2016).

DISCUSSION

This is the first epidemiological study aimed to genotype *Malassezia pachydermatis* isolates causing canine otitis in Colombia. *M. pachydermatis* is an important colonizer of healthy canine skin and mucosae; however, it can cause otitis externa and dermatitis (Crespo et al. 2002, Ribeiro do Prado et al. 2007, Cafarchia et al. 2008, Pulido et al. 2010). In humans, this species has been reported in bloodstream infections, possibly caused by zoonotic transmission (Gueho et al. 1987, Morris et al. 2005, Seyedmousavi et al. 2015). The interest in the study of *M. pachydermatis* has increased in the last decades, given the better understanding of aspects regarding its epidemiology and ecology, as well as the unraveling aspects of the pathogenesis and its role in the developing of the diseases. Nevertheless, many aspects are still unknown.

Several molecular typing methods have been previously reported to resolve the species identification discordances between conventional methods, such as morphological and biochemical methods, commonly used for this purpose (Sugita et al. 2010). These advances have been crucial to progress in the diagnostic field. In our study, average size of the amplified 5.8S rDNA-ITS2 region fragment was similar to that reported by Gaitanis et al. (2002), who obtained an average amplicon size of 483bp for *M. pachydermatis* isolates from dogs lesions diagnosed with otitis. For the 26S rDNA region fragment, our findings agreed with those of Gupta et al. (2004) who reported an average amplicon size of approximately 550bp.

PCR-RFLP is a reliable tool to perform an undemanding identification of *Malassezia* species, having been widely reported in epidemiological studies in human skin diseases (Gupta 2004, Lee et al. 2006, Jang et al. 2009, Sugita et al. 2010, Sosa et al. 2013, Soares et al. 2015). Through the current study, we were able to identify *M. pachydermatis* obtained from otitis externa in dogs. The results obtained in our study for the analysis of the restriction patterns of 5.8S rDNA-ITS2 and 26S rDNA regions with *Alu*I and *Cfo*I, concurred with the previous reports for *M. pachydermatis* (Mirhendi et al. 2005, González et al. 2009).

The digestion of 26S rDNA fragment with *BstF5*I revealed a dimorphic banding pattern. In previous studies, the only reported banding pattern obtained by *BstF5*I restriction on *M. pachydermatis* isolates consisted of two bands of 500 and 70bp (Mirhendi et al. 2005, González et al. 2009). Remarkably, our results showed a new restriction pattern for the digestion of 26S rDNA region with *BstF5I,* this new pattern consisting of four bands was found in most of the isolates included in this study. The *in silico* restriction analysis corroborates this finding giving new insights on this species epidemiology in our country. This could be attributed to a genetic variability in the 26S rDNA region for this species and others in this genus, as it has been proposed by several researchers using diverse molecular tools (Aizawa et al. 1999, Cabañes et al. 2005, Cafarchia et al. 2007, 2011a, Duarte & Hamdan 2010, Wu et al. 2015, Puig et al. 2016). These differences highlight the importance of performing molecular typification for achieving a better identification, and broadening the epidemiological knowledge in animal health (Gupta 2004).

Additionally, different studies have revealed the presence of a high genetic variability into this species with the presence of different genotypes/subgenotypes that can be isolated from domestic and wild animals (Guillot et al. 1997, Cafarchia et al. 2007, 2008, 2011a, Gandra et al. 2008, Álvarez-Pérez et al. 2015, Puig et al. 2016). Here we were able to identify the presence of at least three sequence types (I, IV, V) for *M. pachydermatis* in D1/D2 and ITS regions. Particularly, the two restriction patterns found for 26S rDNA region with *BstF5*I were grouped with different sequence types, suggesting a high diversity of this species. These genotypes can be detected in other

animals such as cats and wild mammals, hence, is important to perform additional analysis including a wide number of isolates from this sources to understand this diversity.

Many other aspects should be explore to have better comprehension about the relation between the presence of this genotypes and a possible connection with the establishment of the disease, such as the relation with bacterial microbiota, pH and fatty acids composition, aspects that have been linked to the adaptation of this species to some anatomical sites of the host due to the differences found in the lipid metabolism of this yeast (Cafarchia et al. 2008, Buommino et al. 2016, Puig et al. 2016, Triana et al. 2017).

CONCLUSIONS

The results revealed the presence of different genotypic variants of *Malassezia pachydermatis* in Colombia.

The existence of different genotypes could be a tool to accurate the clinical management of the species, as well as to understand the ecology and epidemiology of *M. pachydermatis* and contribute with this data at local and global level.

Acknowledgments.- The authors are grateful to "Vicerrectoría de Investigación" of "Pontificia Universidad Javeriana" for financial support (grant ID: PTA 00004585).

Conflict of interest statement.- All authors declare no conflict of interest.

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