Epidemiology of *Malassezia*-Related Skin Diseases

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Core Messages

› This chapter deals with the range of molecular biology methods including PCR-based assays, high-throughput DNA sequence analysis, and the use of real-time PCR in performing studies of skin and environmental community structure and dynamics. It highlights the utility of each molecular biology method in yielding *Malassezia* epidemiological data, the limitations of culture-based methods, and the possible biases that may influence *Malassezia* epidemiological studies. As in any disease or health-impacting event, the frequency and patterns of *Malassezia*-related diseases is examined by descriptive epidemiology. Thus, the chapter includes assessment of patient-related factors such as age, immunological status and gender, and environmental factors such as the geographical area and time of the year that contribute to the analysis of risks from *Malassezia*-induced or *Malassezia* -exacerbated disease. Also, a comprehensive account is given on the *Malassezia* epidemiology in animals and the factors associated with the animal hosts and their skin microenvironment.

3.1 Molecular detection and Identification of *Malassezia* yeasts in the Epidemiological Studies

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3.1.1 Introduction

In recent years, molecular studies have considerably changed the taxonomy of the genus *Malassezia.* The molecularly defined species can still, to some extent, be discriminated

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T. Boekhout et al. (eds.), *Malassezia and the Skin*
DOI: 10.1007/978-3-642-03615-6_3, © Springer Verlag Berlin Heidelberg 2010

using morphological and physiological methods (see Chap. 2.1). Among the earliest molecular characteristics that were used to discriminate between species were analysis of Mol% guanine plus cytosine, and the assessment of DNA relatedness using DNA reassociation techniques [86]. Since the recognition of lipid dependent species, other than *M. furfur* [87], it became clear that molecular approaches are needed for better diagnostics, as well as our understanding of *Malassezia* community dynamics. For instance, 13.8% of isolates identified by phenotypic means were found to be misidentified after molecular re-identification using sequence analysis of the D1/D2 domains of the large subunit ribosomal rDNA (LSU rDNA) and the ITS1+2 regions [103].

Here, we present an overview of the molecular methods that have been applied to detect and identify *Malassezia* species composition on humans and animals. The methods used can be divided into four main approaches: (1) Biotyping using enzymatic methods; (2) Chromosomal analysis using pulsed field gel electrophoresis (PFGE); (3) PCR-based methods; and (4) DNA sequence based methods. It is noteworthy that the PCR- and sequence-based methods used for *Malassezia* biodiversity studies, and those employed to study *Malassezia* community structure on skin and molecular epidemiology are often similar and the distinction is not always clear (see Chap. 2.1).

3.1.2 Biotyping

In biotyping of *Malassezia* isolates, the use of enzyme activities has received little attention. In a Spanish study, 120 isolates from skin were biotyped using Api 20 NE and ApiZym (bioMérieux, Marcy l'Etoile, France) and they could be identified as *M. globosa*, *M. sympodialis*, and *M. restricta* using enzyme activity profiles. Biotype 1 (=*M. globosa*) was catalase positive, esculin negative, and lipase (C14) positive; biotype 2 (=*M. sympodialis*) was catalase positive, esculin positive, and lipase (C14) negative; biotype 3 (=*M. restricta*) was catalase negative, esculin negative, and lipase (C14) negative [7]. In another study, enzyme activities of 33 *Malassezia* isolates belonging to six species were studied using ApiZym. Species-specific differences were observed, and, in general, limited infraspecific variation was noted [130].

Phospholipase, proteinase, hyaluronidase, and chondroitin-sulfatase activities were studied in 30 isolates of *M. pachydermaris* obtained from otic secretions and skin scrapings from dogs. No significant differences in enzyme activities were observed to occur between the isolates [46]. Phospholipase activity, considered to contribute to fungal virulence, has been studied in *M. pachydermatis* and differences were observed in phospholipase activity between isolates obtained from lesional and nonlesional skin [32, 37, 38].

Multilocus enzyme electrophoresis of 13 enzymes from 52 isolates of *M. pachydermatis* yielded 27 electrophoretic types (ETs). Interestingly, a correlation was observed between ETs and host specificity. Moreover, the results indicated that genetic exchange may occur between the various ETs, thus suggesting the presence of sexual recombination in this species [138].

3.1.3 Electrophoretic Karyotyping

Electrophoretic karyotyping using PFGE demonstrated a heterogeneity in chromosomal patterns among *Malassezia* yeasts. Various techniques have been applied, e.g., LKB

Table 3.1 Genome size of some *Malassezia* species based on PFGE [13, 15]

Species	Number of chromosomes	Genome size (Mb)
M. furfur type I	7–8	8.5
M. furfur type II	10–11	14
M. globosa	8	8.5–8.9
M. obtusa	6	7.4
M. pachydermatis	5	6.7–9.3
M. restricta	9	8.0
M. slooffiae	7	8.6
M. sympodialis	7	6.4–7.8

Pulsaphor and CHEF Dr-II (Biorad), with the latter technologically being more reliable due to the absence of mechanically moving parts. Using Pulsaphor, isolates of *M. furfur* were found to have seven chromosomes, which could separate them in three groups that correlated with the three morphological types that were distinguished before [115, 136].

CHEF analysis of *M. pachydermatis* isolates and lipophilic *Malassezia* spp. [13, 14] demonstrated that the karyotypes of *M. pachydermatis* were similar to each other and contained five chromosomes, whereas among the lipophilic isolates four different patterns could be discerned. In the latter study [15], the lipophilic species with different karyotypes could be linked to the species described by Guého et al. [87]. One of the main conclusions was that *Malassezia* species showed limited intraspecific chromosomal length polymorphism (CLP). The observed heterogeneity in *M. furfur* could be related to the presence of putative hybrids between different AFLP genotypes of that species (T. Boekhout, unpublished observation). Another remarkable feature of the *Malassezia* genomes is their small size ranging from 6.4 to 14 Mb (Table 3.1) [15, 208]. The genome size of *M. globosa* of 8.5–8.9 Mb as estimated by PFGE analysis agreed with that based on whole genome sequencing, namely 9 Mb [208]. The latter analysis also suggested that the genome is haploid as the level of polymorphism present was found to be very low (>0.0004%). PFGE has also been used to confirm phenotypically identified *Malassezia* isolates. In a study of 220 isolates from affected skin of animals and humans, 217 were identified as *M. pachydermatis* and three as *M. sympodialis*. The majority of the *M. pachydermatis* isolates contained six chromosomes and 17 had seven. Interestingly, two isolates obtained from both ears of a single dog revealed 6 and 7 chromosomes, respectively [182]. The *M. sympodialis* isolates also showed some CLP. On the basis of these studies, PFGE may be used to identify *Malassezia* species, but the method is time consuming and methodologically demanding. Moreover, karyotypes of the recently described species (e.g., *M. caprae, M. dermatis, M. equina, M. japonica, M. nana* and *M. yamatoenis*) have not yet been analyzed.

3.1.4 PCR-Based Methods

Many genotypic methods used to discriminate between isolates of *Malassezia*, or to identify them, are based on PCR. Among these are genotypic methods that generate a banding pattern

per strain that can be compared with other such banding patterns available (e.g., RAPD, AFLP, PCR-RFLP). Alternatively, amplicon lengths may be compared (e.g., *t*FLP), or the migration behavior of single stranded DNA is used to infer relationships between isolates in DGGE or SSCP analyses (see below). Species-specific PCR probes have been developed for diagnostics. These can be used in individual PCRs or, technically more advanced, they can be hybridized to probes bound to latex beads (e.g., Luminex technique) thus allowing further, high-throughput automation for use in large-scale epidemiological studies. Furthermore, real-time PCR has been used to study *Malassezia* community structure and dynamics.

3.1.4.1 Random Amplification of Polymorphic DNA (RAPD) and DNA Fingerprinting

RAPD typing of *Malassezia* isolates has been used in various studies. The epidemiological analysis of an outbreak at a neonatal ward caused by both *M. furfur* and *M. pachydermatis* [201] was among the first. This study used prokaryotic repeat consensus primers based on enterobacterial repetitive intergenic consensus (ERIC) or repetitive extragenic palindromic (REP) motifs. The outbreak isolates showed identical banding patterns if compared with the reference strains. Hence, it was concluded that the outbreak was caused by a single genotype and most probably a single strain, despite occasional subtle differences that were observed by PFGE [13]. Apparently, ERIC and REP PCR-typing showed better epidemiological resolution than PFGE. It is important to realize that PFGE is not directly comparable with ERIC and REP PCR-typing, as each method targets different elements of the *Malassezia* yeast genome. In another study of an outbreak in a neonatal ward caused by *M. pachydermatis*, RAPD analysis showed the presence of genetically distinct isolates involved in the outbreak [44], and similar observations were made in a *M. pachydermatis* outbreak in a US-based hospital [199]. The use of RAPD typing for taxonomic and henceforth epidemiological purposes has also been investigated [15]. Representatives of all known species at that time were investigated by 20 decamer primers. Primers OPA 02, OPA 04, OPA 05, and OPA 13 (Operon Technologies, Alameda, CA, USA) gave best resolution. All species could be discriminated, but straightforward analysis was hampered by the presence of considerable intraspecific variation [15]. Similar results were obtained in an epidemiological study of isolates from skin, where *M. sympodialis* showed the greatest homogeneity [41]. Within 55 isolates of *M. pachydermatis* obtained from various domestic animals and body sites, RAPD discriminated four genotypes; one of them occurred in various animals (cats, horse, goat, pig) and the other three were observed only in the external ear canal of dogs; different genotypes could occur on a single animal or even at a single body site [40]. Other studies on *M. pachydermatis* also demonstrated genetic heterogeneity [114, 126]. In an extensive study of 210 isolates using RAPD, morphology, catalase and β-glucosidase activities, lipid and carbohydrate growth requirements, and tryptophan-dependent pigment synthesis [114], it was concluded that extensive phenotypic and genotypic diversity occurs within the species. A single genotype was found to be correlated with pigment production and the authors proposed to use this observation as a possible screening to search for this character in a larger number of isolates. In a study on the presence of genetic diversity in *M. furfur*, 47 isolates from either pityriasis versicolor (PV) patients, seborrheic dermatitis (SD) patients, or SD in AIDS patients, RAPD patterns were found to correlate with these three patient

categories. On the basis of this observation, the authors suggested that RAPD is a useful epidemiological tool [76]. M13-DNA fingerprinting of 42 globally collected isolates of *M. furfur* yielded six genotypic clusters that correlated with the underlying skin condition and the hosts' geographic origin. Isolates from Scandinavia, Greece, Bulgaria, and China showed a propensity for geographic clustering. The authors concluded that *M. furfur* may be a good candidate species to investigate human phylogeography patterns [74].

3.1.4.2 Restriction Fragment Length Polymorphism (RFLP) Analysis

Digestion of PCR amplicons has shown to be useful for the separation of *Malassezia* species [71, 72, 82, 96, 114]. Sequence polymorphisms in the target gene or DNA region of choice can be made visible by the use of restriction enzymes. The ribosomal DNA, including the large subunit (LSU) rDNA and internally transcribed spacer (ITS) regions have been DNA targets explored for diagnostics [96, 100, 103]. Using the *Malassezia* specific primers Malup (5'-AGC GGA GGA AAA GAA ACT-3') and Maldown (5'-GCG CGA AGG TGT CCG AAG-3'), the LSU rRNA gene could be amplified resulting in a fragment between 541 and 579 bp [96]. All seven species recognized at that time could be discriminated by digestion of the amplicon using the restriction enzymes BanI, HaeII, and MspI. Moreover, no intraspecific variation was observed, rendering this method a *Malassezia*-specific identification tool. In addition, species in mixed samples of *M. globosa* and *M. sympodialis*, or *M. furfur* and *M. sympodialis*, respectively, could be discriminated [96]. Later, this method was extended to separate 11 species [159] (Table 3.2). In another RFLP study, five out of seven species could be discriminated using AvaI digestion of the LSU rDNA and digestion of the ITS regions by EcoRI and NcoI [100]. However, *M. globosa* and *M. restricta* could not be discriminated using the latter enzymes, and, therefore, an alternative hot start touch-down PCR of the

Table 3.2 RFLP profile of eleven *Malassezia* species based on three restriction endonucleases

Species	ITS 3/4 PCR product (bp)	AluI	BanI	MspAI
Malassezia dermatis	416	NRS	186, 230	192, 85, 109, 30
Malassezia furfur	557	306, 251	389, 168	525, 32
Malassezia globosa	477	221, 16, 240	NRS	447, 30
Malassezia japonica	528	394, 134	183, 199, 146	498, 30
Malassezia nana	428	NRS	188, 240	286, 110, 32
Malassezia obtusa	554	NRS	396, 158	NRS
Malassezia pachydermatis	529	412, 117	NRS	499, 30
Malassezia restricta	463	NRS	186, 277	432, 31
Malassezia slooffiae	505	385, 120	NRS	472, 33
Malassezia sympodialis	420	NRS	NRS	281, 109, 30
Malassezia yamatoensis	470	NRS	NRS	NRS

NRS no restriction site

β-tubulin gene was needed for a proper identification of these two species. Gaitanis et al. [71] developed a PCR-RFLP aiming to identify *Malassezia* species present in skin scales. For DNA extraction, a cetyltrimethylammonium bromide (CTAB) protocol was employed. Panfungal primers targeting the ITS, namely ITS 1 (5'-TCC GTA GGT GAA CCT GCG G-3'), ITS 3 (5'- GCA TCG ATG AAG AAC GCA GC-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), were used to amplify the ITS regions. In case of a weak amplification result using ITS 3 and ITS 4 primers, the primer combination ITS 1 and ITS 4 was used, followed by a nested PCR using primers ITS 3 and ITS 4. Length estimates of the amplicons generated by primers ITS 3/ITS 4 discriminated between *M. furfur, M. sympodialis, M. globosa*, and *M. pachydermatis*. However, using standard agarose conditions, *M. restricta* could not always be separated from *M. slooffiae* or *M. pachydermatis.* Digestion of the amplicons by AluI and HinfI discriminated between all seven species included in the study, namely *M. furfur*, *M. obtusa*, *M. slooffiae*, *M. restricta*, *M. sympodialis*, *M. globosa*, and *M. pachydermatis*. The relative sensitivity was found to be 10±5 cfu. Subsequently, 11 *Malassezia* species using pure culture were discriminated (Table 3.2) through restriction of PCR amplicons that were generated with the ITS 3/4 primers [72]. PCR-RFLP of the LSU-rDNA using CfoI and BstF51 discriminated among 11 species, and was successfully applied to clinical isolates [139]. PCR-RFLP has also been used to identify larger numbers of clinical isolates [39, 212] or isolates from animals [160]. Importantly, tracing an outbreak of *M. pachydermatis* in an intensive care unit was possible using RFLP analysis. Analysis of isolates obtained from neonates, health care workers and their pet dogs demonstrated that transmission occurred from a pet dog via the health care worker to the IC patients [42] (see Chaps. 3.3 and 8).

3.1.4.3 Amplified Fragment Length Polymorphism (AFLP™) analysis

AFLP, a DNA fingerprinting technique based on the selective amplification of restriction fragments from a digest of genomic DNA [202], has been successfully applied to understand the genetic relationships among isolates of *Malassezia* [31, 103, 199]. In AFLP analysis, all lanes contain a size standard and, therefore, the method is, at least theoretically, less prone to experimental variation than, for instance, RAPD analysis. Practically, variation has been noted to occur between separate runs, especially when a time interval occurs between runs, thus rendering an easy comparison sometimes difficult. The same may be true for interlaboratory comparisons, although the variation observed may be less than in RAPD analyses. In case of critical studies, we therefore recommend running the experiment(s) in a single run, in order to avoid experimental bias as much as possible.

Using AFLP all species recognized could be separated [31], the genetic diversity within species was illustrated, and in some species distinct genotypes could be discerned [103, 199] (Fig. 3.1). In *M. furfur*, four [199] and in a more extended study eight [103] AFLP groups could be recognized. Probably the most significant observation was that 80% of the isolates of AFLP genotype 4 came from deep or mucosal sources, such as urine, blood, tracheal secretion, nasal smear, and feces [199]. In a more extensive sampling, genotype 1 isolates came from chest and back, genotypes 2 and 8 contained animal isolates, genotypes 4 and 5 originated from deep body sites, and isolates from genotypes 1 and 6 seemed to have a preference for

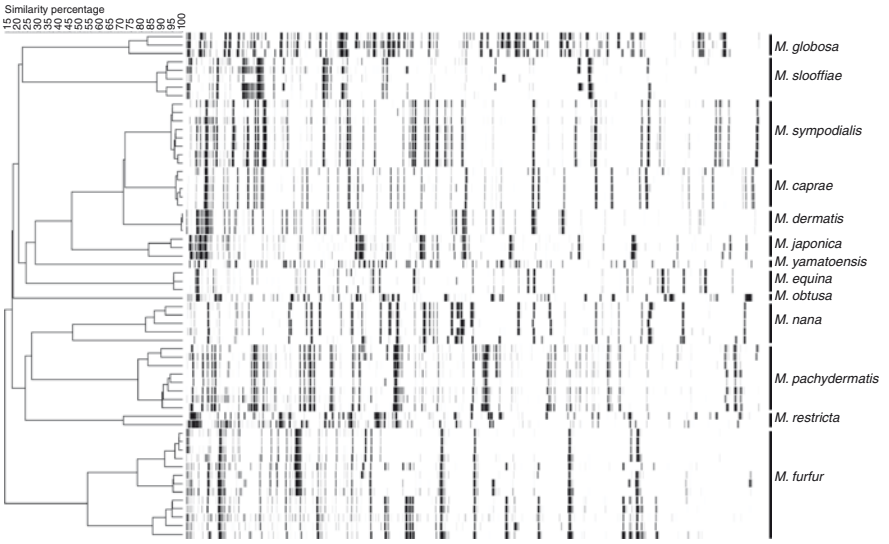

Fig. 3.1 Genotypic diversity among *Malassezia* species determined by amplified fragment length polymorphism (AFLP). The AFLP patterns were generated using the selective primers EcoR1A and Mse1G as described [199], and the tree was created with Bionumerics (Applied Math, Belgium) using curve-based Pearson correlation in combination with single linkage

neonates. The distribution of AFLP genotypes between North America and Europe was found to be significantly different [103]. AFLP seems to be the tool of choice for genotypic analyses when detailed genotypic information is needed and many isolates are available.

3.1.4.4 Terminal Fragment Length Polymorphism (*t*FLP) Analysis

The size of ITS 1 and ITS 2 amplicons can potentially be used to discriminate between species (see also above under RFLP [71]). Similar approaches have been described to identify other clinically important yeasts, such as *Candida* spp. [56]. This method was further developed using capillary detection of the amplicon size, making the method suitable for automation. This so-called terminal fragment length polymorphism (*t*FLP) used the amplicon lengths of both the ITS 1 and ITS 2 for discrimination of *Malassezia* species [80]. The method can also be used on noninvasively acquired swab samples and uses only three ITS-based primer sets, and therefore, minimizes the potential bias related to amplification efficiency. It is sensitive enough to detect *Malassezia* with as few as 100 cells per sample, either dosed directly onto the swab for extraction control or for data collection from 1 cm^2 of skin surface sample. This method involves isolation of fungal DNA, followed by nested PCR of the ribosomal gene cluster and amplification with ITS 1 and ITS 2 specific fluorescently labeled primers. The resulting terminally-labeled products are analyzed for fragment length on a fluorescent DNA sequencer. The amplifications are carried out with universal fungal primers [80], and therefore the methodology should be broadly applicable to other fungal species. The ITS 1 and ITS 2 amplifications are carried out individually and combined for

final analysis. The primary advantage of this method is that the resultant sample contains only two labeled fragments per species, allowing analysis of complex communities. All *Malassezia* species can be differentiated by length polymorphisms, including multiple genotypes per known species. Results obtained for standards and mixtures of standards showed that all known *Malassezia* genotypes can be identified in a single amplification reaction based on unique fragment lengths, thus eliminating the need for restriction analysis. Results from this study also showed that *t*FLP is capable of reproducibly assessing the *Malassezia* species present in complex mixtures and clinical samples. In an analysis of subjects assigned for different grades of dandruff, i.e., a comparison between persons with composite adherent flaking score (ASFS) < 10 (low dandruff) and those with a composite ASFS > 24 (high dandruff) showed that in both human populations *M. globosa* and *M. restricta* were predominant species in samples from scalp, with both species being more prominent in high ASFS subjects. Importantly, *M. furfur* was not detected [80]. The authors concluded that both *M. globosa* and *M. restricta* are involved in causing dandruff.

3.1.4.5 Denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) analysis

DGGE and SSCP are methods that can discriminate among PCR amplicons that have a similar size, and, hence cannot be separated using standard electrophoretic techniques. The principle is that the tertiary conformation of single stranded DNA, which determines the running distance in a gel, depends on the nucleotide sequence.

DGGE uses a denaturing gel, e.g., containing a urea gradient, to separate both strands of the PCR amplicon [143, 144], whereas SSCP separates, in a nondenaturing gel, heat separated single stranded DNA that has been cooled down quickly [79]. Both methods require dedicated equipment, and as the running behavior in the acrylamide gels depends on the experimental conditions, results are not always straightforward to interpret. It seems that results based on SSCP are less prone to experimental variation, and, in addition, running conditions do not need to be optimized for each new PCR. For these reasons, DGGE analysis has only rarely been applied to differentiate *Malassezia* strains [199]. All seven species included in the study could be distinguished, and two isolates of each *M. furfur* and *M. pachydermatis* were found to have identical banding patterns.

PCR-based SSCP allows detection of point mutations in PCR amplicons, e.g., those of the widely used ITS regions. Commonly used primers (e.g., ITS 1 and ITS 2) allow efficient amplification. Separation of single stranded DNA that is obtained after denaturation of the amplicons at e.g., 94°C followed by immediate snap-cooling on ice or a freezing block is performed on a nondenaturing acrylamide/bis-acrylamide gel at low temperatures (e.g., 11°C) (for a detailed protocol see ref. [79]). Five genetic subtypes were identified in *M. globosa* isolates obtained from PV and SD by SSCP using the ITS 1 spacer [73]. SSCP subgroup A was found to be correlated with the presence of more extensive lesions. Twelve isolates of *M. sympodialis* did not show any SSCP ITS subtypes [73]. Among 185 isolates of *M. pachydermatis* obtained from diseased and nondiseased skin of 30 dogs and from various body sites, eight different ITS 1 genotypes could be discriminated by SSCP that were also confirmed by sequencing analysis [38]. One genotype (i.e., genotype B) was observed to

occur mainly on healthy skin. Fifty percent of the dogs contained multiple ITS subgenotypes at different body sites [38]. Next to SSCP separation of ITS amplicons, the method has also been used to separate amplicons based on the chitin-synthase-2 (CHS-2) gene [35].

3.1.4.6 Detection by Luminex Technology

Sensitive detection of *Malassezia* species, either alone or in mixed populations, has been made possible using a bead suspension array that combines the specificity and reliability of DNA-based probes and the speed and sensitivity of the Luminex analyzer [57]. Probes based on sequence divergence in the D1/D2 domains of the LSU rDNA and the ITS spacers are covalently bound to unique sets of fluorescent beads allowing detection of the specific probes using a red laser (636 nm), and, hence, identification of the species present. After hybridization, the biotinylated PCR amplicon is detected using a green laser (532 nm) by addition of a fluorochrome coupled to a reporter molecule (e.g., streptavidin). Probes covered either a narrow or a broader taxonomic range, and discriminated between species, groups of species, or all representatives of the genus. This allowed detection of species in a multiplex and high-throughput format [57]. The hybridization assay could discriminate between sequences that only differed in 1 or 2 bp, but specificity depends on the position of the mismatch as well as the probe design. Accurate identification of species is achieved using a multiplex format, thus allowing the analysis of mixed clinical samples. Interestingly, the method could even use direct amplification from a pin-head-sized portion of cells of *M. pachydermatis*, thus without the need to isolate DNA. If this is true for the other species as well, this method may be used to analyze the *Malassezia* community structure fast and reliably [57]. However, one may expect considerable differences in the efficiency of the PCR performance between the various species when omitting DNA extraction, thus complicating the analysis.

3.1.4.7 Epidemiological Investigations by Real-time PCR

PCR approaches, such as real-time PCR as well as *t*FLP (see Sect. 3.1.4.4 above) have been used to investigate the presence of *Malassezia* species without the need to culture isolates. Detection of *Malassezia* species from clinical samples has been performed by real-time PCR using a nested PCR approach targeting the intergenic spacer (IGS) or the internal transcribed spacer (ITS) [5, 188]. In a series of studies, the number of *Malassezia* cells present on healthy and diseased skin was quantified using Taq-Man probes based on D1/D2 LSU rRNA gene sequences (Fig. 3.2). LSU rDNA was amplified using primers pITS (5'-GTCGTAACAAGGTTAACCTGCGG-3') and NL4 (5'-GGTCCGTGTTTCAA GACGG-3'). After insertion in a plasmid, the plasmid DNA was quantified using a NanoDrop ND-1000 spectrophotometer. For real-time PCR, 200 nm of each primer and 250 mM of the Taq-Man primer were used (Table 3.3).

Ten to the power of two to ten to the power of nine (10^2–10^9) copies of plasmid DNA of all 11 species tested could be detected using the primers Mala-F, Mala-R, and Mala-MGB. Importantly, cross reactivity was neither observed against DNA from a variety of clinically relevant yeast species, e.g., belonging to *Candida*, *Cryptococcus*, and *Rhodotorula*, nor from

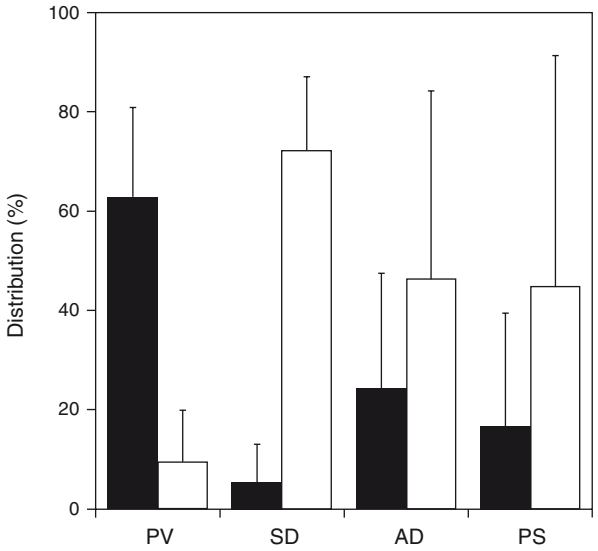

Fig. 3.2 Relative occurrence of *Malassezia globosa* (black bar) and *M. restricta* (*empty bar*) as detected by real-time PCR in pityriasis versicolor (PV), seborrheic dermatitis (SD), atopic dermatitis (AD) and psoriasis (PS)

Table 3.3 Primers used in real-time PCR [193]

Universal *Malassezia* primers	
Mala-F (forward)	5'-CTAAATATCGGGGAGAGACCGA-3'
Mala-R (reverse)	5'-GTACTTTTAACTCTCTTTCCAAAGTGCTT-3'
Mala-MGB (probe)	FAM-TTCATCTTTCCCTCACGGTAC-MGB
***M. globosa* primers**	
M.glob-F (forward)	5'-GGCCAAGCGCGCTCT-3'
M.glob-R (reverse)	5'-CCACAACCAAATGCTCTCCTACAG-3'
M.glob.MGB (probe)	FAM-ATCATCAGGCATAGCATG-MGB
***M. restricta* primers**	
M.rest-F (forward)	5'-GGCGGCCAAGCAGTGTTT-3'
M.rest-R (reverse)	5'-AACCAAACATTCCTCCTTTAGGTGA-3'
M.rest-MGB (probe)	FAM-TTCTCCTGGCATGGCAT-MGB

bacteria. Using real-time PCR, it could be demonstrated that the number of *Malassezia* cells present in the head and neck of 34 AD patients was 12.4 times higher than that on the trunk and 6.8 times higher than that present on the limbs [193]. Both *M. globosa* and *M. restricta* occurred on all AD patients investigated, and these species caused approximately 80% of the colonization by *Malassezia*. In another study of AD patients, it was observed that *M. restricta* and *M. globosa* were the dominant species in adults [188], whereas *M. restricta* occurred dominantly in children with AD [197]. *M. dermatis*, *M. sympodialis*, *M. furfur*, *M. obtusa*, and *M. yamatoensis* were detected in 2–8% of the subjects, and *M. japonica* and *M. slooffiae* were not detected. Using real-time PCR, the colonization rate by *Malassezia* was found to be 2.4-fold

higher in adults with AD than in children with this disease. In children and adults, colonization by *M. restricta* was, respectively, 3.5 and 1.5 times higher than that of *M. globosa* [197].

In a similar study on the presence of *Malassezia* species occurring on the skin of 31 SD patients (i.e., 31 lesional and 27 nonlesional samples), *M. globosa* and *M. restricta* were observed in 93.5 and 61.3% of the SD patients, respectively [195]. *M. dermatis*, *M. slooffiae*, and *M. sympodialis* were detected in 20–30% of the patients, and the remaining species occurred in less than 10%. In both lesional and nonlesional skin, *M. globosa* and *M. restricta* occurred at 70.4 and 55.6%, respectively, but both types of skin did not differ in species composition. In a comparison between AD, SD, PV, and healthy skin, the detection rate of all species was the highest in AD patients with 4.1 ± 1.9 species occurring [195]. Using real-time PCR, lesional skin was found to harbor three times as many *Malassezia* cells when compared with nonlesional skin. *M. restricta* was by far the dominant species in both lesional- and nonlesional skin with an occurrence of $72.3 \pm 15\%$ in lesional skin and $63.6 \pm 16.4\%$ in nonlesional skin, whereas the occurrence of *M. globosa* was $5.1 \pm 7.9\%$ and 6.5 ± 6.3 %, respectively (Fig. 3.2). In conclusion, these analyses suggested that *M. restricta* is the dominant species occurring on SD patients. Genotypically, eight IGS sequence types occurred among the isolates of *M. globosa*, and five of them (i.e., IGS types II, III, IV, V, VI) occurred on SD patients only, whereas type VII occurred on healthy skin, and types I and VIII occurred on both skin from SD and healthy individuals. In *M. restricta*, two main IGS genotypes were apparent, and the isolates of cluster II originated from SD patients and those of cluster I came from both SD and healthy individuals [195]. The authors concluded that SD is a *M. restricta* dominated disease.

In a study of 20 psoriatic patients, real-time PCR analysis indicated that *Malassezia* spp. colonized the trunk and head more heavily than the limbs [196]. *M. globosa* and *M. restricta* occurred on almost all patients with psoriasis, namely 98.0% and 91.8% of lesional skin, and 100% and 91.7% of nonlesional skin, respectively. *M. restricta* was found to be the dominant species in both lesional and nonlesional skin, with a presence of 46.9% and 50.3%, respectively, whereas *M. globosa* occurred only in 12.6% and 8.2% of these samples, respectively [196]. Interestingly, psoriatic patients with normolipidaemia showed greater colonization by *Malassezia* yeasts, including *M. restricta* and *M. globosa*, than patients with hyperlipidaemia. The reason for this is not known, but the authors suggested a possible relationship with the observed presence of atrophic sebaceous glands and reduced lipid production as observed in hyperlipidaemic transgenic mice. Second, it was suggested that antifungal agents might be more effective in patients with normolipidaemia [196].

Paulino et al. [158] investigated the dynamics of the *Malassezia* community structure in time on six healthy skin and two psoriatic skin samples using real-time PCR with dual-labeled probes based on the 5.8 S rRNA gene and the ITS2 spacer that targeted either each of six species or the entire genus. A good correlation was observed between data obtained by real-time PCR and those obtained in a clone library study [157, 158]. *M. restricta* occurred in 57–100% and 7–99% of skin samples from healthy patients and psoriasis patients, respectively, and *M. globosa* was the dominant species, but no differences were observed between lesional and nonlesional skin. *M. sympodialis* was detected exclusively on the upper back of healthy individuals, but only in low amounts (i.e., 10^2–10^5-fold less than *M. restricta*). When comparing the community structure during a 4-month sampling period in 1-month intervals, little variation in the *Malassezia* communities was observed [158].

Lesional skin and nonlesional skin of psoriasis patients revealed that all nine species, for which *Taq*-Man probes were available, could be detected in 22 patients. Detection rates of *M. globosa, M. restricta*, and *M. sympodialis* were 82, 96, and 64%, respectively. No differences occurred between lesional and nonlesional skin, and no species-specific colonization dependent on age, gender, body site, severity of psoriasis, or treatment was observed [5]. The skin of both psoriasis and AD patients showed a greater diversity in *Malassezia* species as compared with normal skin, with an average 3.7±1.6, 4.1±1.9, and 2.8±0.8 species occurring, respectively.

In a study on PV, all PV lesions from 49 patients were found to be positive for *Malassezia* spp. and *M. globosa* and *M. restricta* predominated in 93.9% of the cases [142]. Other species as *M. sympodialis* (34.6%), *M. dermatis* (24.4%), *M. furfur* (10.2%), *M. obtusa* (8.1%), *M. japonica* (6.1%), and *M. slooffiae* (4.1%) were less frequently detected. In lesions that showed hyphal growth of *Malassezia* cells, which is considered an important factor contributing to PV, only *M. globosa* could be detected. Therefore, the authors concluded that PV is mainly caused by *M. globosa* [141].

These molecular studies suggest that *M. globosa* and *M. restricta* are the most important species involved in the different skin disorders, AD, SD, PV, and probably psoriasis as well. Therefore, emphasis to understand the pathophysiology of *Malassezia*-mediated skin disorders should focus on these two species.

3.1.5 DNA Sequence Analysis

Nucleotide sequence analysis of the ribosomal DNA locus has fundamentally contributed to our current understanding on the biodiversity of *Malassezia* yeasts. More recently, other genes have also been explored for their ability to contribute in understanding the species boundaries within the genus.

3.1.5.1 Ribosomal DNA Sequences

The ribosomal RNA (rRNA) genes (also referred to as rDNA, but this also includes the IGS regions) has been widely used for the identification of microorganisms, including clinically relevant yeasts. Fig. 3.3 shows the primary structure of the *Malassezia globosa* rRNA genes and spacer regions. Fungal rRNA genes occur in tandem repeats, with each repeat encoding 18S (or small subunit, SSU), 5.8S, 26S (or large subunit, LSU), and 5S rRNA genes. Two

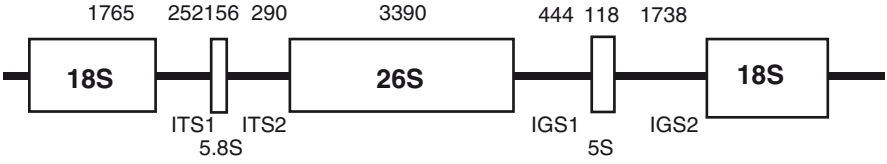

Fig. 3.3 Primary structure of the rDNA locus. On top are the approximate sizes in nucleotides of the various parts as they occur in *Malassezia* yeasts, in the boxes the major rRNA genes are indicated, and below are the smaller 5.8S and 5S rRNA genes and the spacer regions

spacer regions exist in each repeat, namely the Internal Transcribed Spacer (ITS) regions located between SSU and LSU, and the InterGenic Spacer (IGS) region located between LSU and SSU. The four subunits have more or less constant lengths regardless the species concerned as follows: SSU 1800 bp, 5.8S 160 bp, LSU 3,500bp, and 5S 120 bp. The rRNA genes can be compared among phylogenetically distant species because the sequences are highly conserved, but phylogenetically closely related species or isolates can be compared as well because of the presence of more variable stretches of DNA, such as the ITS and IGS spacers (Table 3.4). At present, sequences of the rRNA genes and ITS regions of almost all human- and animal pathogenic fungi have been deposited in GenBank.

To be useful as a species-specific diagnostic tool, DNA sequences must be evaluated and compared with other criteria, such as phenotype. In general, conspecific strains have identical (or highly similar) sequences, whereas different species have divergent sequences [186, 187]. The domains 1 and 2 (i.e., the D1/D2 domains) of the LSU rRNA gene, which is the most variable region of LSU, and the ITS 1+2 regions are useful for the identification of unknown fungal isolates. Peterson and Kurtzman [161] and Sugita et al. [186] suggested that conspecific strains have a similarity of 99% or higher in both these regions. In practice, almost all pathogenic fungi can be identified following this approach. Sequencing of rDNA is relatively straightforward because universal PCR primers are available. PCR primers used to amplify the ITS region, including the 5.8S rRNA gene, and the D1/D2 domains of the LSU rRNA gene are listed in Table 3.5. Other primer sequences have also been used for amplification, but those designated by White et al. [206] and O'Donnell [154] are the most commonly used. Primers ITS1, ITS4, NL1, and NL4 are useful to amplify rDNA of all fungi. Usually, a nested approach is followed, in which approximately 1.2–1.3 kb of the ITS and D1/D2 LSU is amplified using primers ITS1 (forward) and NL4 (reverse). Subsequently, a sequencing PCR is performed targeting either the ITS regions or the D1/D2 domains of the LSU rRNA gene, or both, using primers ITS1 and ITS4 or NL1 and NL4, respectively.

Guillot and Guého [90] were the first to investigate the usefulness of LSU rRNA sequences to address species diversity in *Malassezia*. This study clearly revealed the

Table 3.4 Taxonomic resolution of each subunit or spacer region in the ribosomal DNA unit

Subunit of spacer region	Class	Family	Genus	Species	Strain
SSU	———	———	———	———	
D1/D2	———	———	———	———	
ITS			- - - -	———	
IGS				———	———

Table 3.5 PCR primers used for D1/D2 LSU rRNA gene and ITS spacer sequence analysis

Subunit or spacer region	Names of primers	Sequences	Annealing temperature
D1/D2 LSU	NL1 (forward)	GCATATCAATAAGCGGAGGAAAAG	65.3 °C
	NL4 (reverse)	GGTCCGTGTTTCAAGACGG	65.5 °C
ITS region	ITS1 (forward)	TCCGTAGGTGAACCTGCGG	68.4 °C
	ITS4 (reverse)	TCCTCCGCTTATTGATATG	57.6 °C

presence of additional species of lipid-dependent *Malassezia* species, next to *M. furfur*, which were subsequently described as *M. globosa*, *M. obtusa*, *M. restricta*, and *M. slooffiae* [87]. Subsequent studies demonstrated the presence of additional species, such as *M. caprae*, *M. dermatis*, *M. equina*, *M. japonica*, *M. nana* and *M. yamatoensis* (see Chap. 2.1). The D1/D2 regions of the LSU rRNA gene, which is approximately 600 bp in length, is the most variable region in the LSU, and generally useful to identify *Malassezia* species [103]. The amplicon length is identical across all species, and the within-species sequence similarity is above 99% for these *Malassezia* species (Table 3.6).

The length of the ITS region differs between species, whereas its length among strains of a species is usually identical, and this can be used as an identification tool (see also RFLP and *t*FLP, above). Some *Malassezia* species, however, are an exception to this. For instance, the *M. globosa* ITS1 region ranges from 237 to 266 bp in length and the within-species DNA sequence similarity is 88% (Table 3.6). The *M. restricta* ITS1 region ranges from 210 to 261 bp in length showing 73% within-species similarity (Table 3.6). Of the 13 *Malassezia* species, *M. caprae*, *M. dermatis*, *M. japonica*, *M. obtusa*, and *M. yamatoensis* do not show infraspecific ITS diversity. The nucleotide order of the ITS regions turned out to be useful for the identification of *Malassezia* strains as well as to infer their phylogenetic relationships [129].

In general, most *Malassezia* species can be identified using the D1/D2 LSU rRNA gene sequence only. Caution is needed, however, as some *Malassezia* species, namely *M. sympodialis* and the phylogenetically closely related species *M. caprae*, *M. dermatis*, and *M. equina,* could not easily be identified following this approach (Table 3.7). *M. caprae* and *M. equina* were separated from *M. sympodialis sensu lato* based on phylogenetic analyses [30, 31], but they also differ phenotypically. D1/D2 LSU sequences of these four species show

Table 3.6 Summary of the ITS1, ITS2, and D1/D2 LSU rDNA sequences of *Malassezia* spp

Species	ITS1		ITS2		D1/D2 LSU
	Length (bp)	Similarity (%)	Length (bp)	Similarity (%)	Similarity (%)
Malassezia caprae	164	100	231	100	100
Malassezia dermatis	161	100	233	100	100
Malassezia equina	162–164	>99	232	100	100
Malassezia furfur	209–210	>95	370	100	>99
Malassezia globosa	237–266	>88	278–290	>91	>99
Malassezia japonica	208	100	337	100	100
Malassezia nana	182–186	>97	241–242	>98	>99
Malassezia obtusa	213	100	367	100	100
Malassezia pachydermatis	186–187	>94	328–344	>94	>99
Malassezia restricta	210–261	>73	276	>99	>99
Malassezia slooffiae	195–196	>99	313–316	>99	100
Malassezia sympodialis	162–163	100	234–235	100	100
Malassezia yamatoensis	176	100	298	100	100

Table 3.7 DNA sequence similarity of rDNA units among the related species *M. sympodialis*, *M. caprae*, *M. dermatis*, and *M. equina*

Subunit or spacer region	Species	*M. caprae*	*M. dermatis*	*M. equina*
D1/D2 LSU	*M. dermatis*	98	X	98
	M. equina	99	98	X
	M. sympodialis	99	99	99
ITS1	*M. dermatis*	93	X	85
	M. equina	82	85	X
	M. sympodialis	88	90	85
ITS2	*M. dermatis*	95	X	91
	M. equina	91	91	X
	M. sympodialis	91	91	88

more than 98–99% sequence similarity and they share 82–93% and 88–95% sequence similarity in the ITS1 and ITS2 regions, respectively (Table 3.7). Therefore, DNA sequencing of both the ITS regions and the D1/D2 domains of the LSU rRNA gene is recommended to identify isolates that may belong to *M. sympodialis* and related species. In practice, it may, therefore, be best to use both these rDNA regions to identify isolates of unknown identity.

The more variable IGS regions are less suitable as an identification tool of fungal species. Sequence analysis of the IGS regions has, however, been applied to assess species diversity within *Malassezia* and this turned out to be particularly useful to study strains genotypically as an epidemiological tool [189–192].

Recently pyrosequencing, yielding c. 30 bp fragments of the ITS1+2 regions and the 5.8S rDNA, has been used to identify *M. slooffiae*, *M. pachydermatis*, and *M. sympodialis* [140]. However, species of *Trichosporon* and some members of *Cryptococcus,* including members of the *C. neoformans* complex, and some related *Bullera* species could not be identified. *Malassezia* yeasts have also been identified with the MicroSeq D2 LSU ribosomal DNA sequence kit [110]. Both these latter methods need further evaluation before they can be implemented in routine diagnostics of *Malassezia* yeasts in the clinical laboratory.

3.1.5.2 Other Genes

Analysis of partial sequences of the mitochondrial Large subunit ribosomal RNA (mtLrRNA) that were generated with primers ML7 (5'-GACCCTATGCAGCTTCTACTG-3') and ML8 (5'-TTATCCCTAGCGTAACTTTTATC-3') discriminated between all seven species studied [209]. Two loci of the chitin-synthase 2 (*CHS2*) gene and the ITS 1 region have been used to genotype isolates of *M. pachydermatis* obtained from dogs and cats [2, 3, 38, 117]. In a sample of 185 dogs, three *CHS2* genotypes and eight ITS1 genotypes were distinguished. Two ITS1 types (namely A_{11} and B_{11}) occurred on all skin sites investigated, whereas one type (C_{12}) was associated with specific body sites [38]. Analysis of partial sequences of the RNA polymerase

subunit 1 (*RPB1*) has been used to assess biodiversity within the *M. sympodialis* clade that also included *M. nana* [31]. The authors, however, were unable to easily amplify the *RPB1* gene in all species and, therefore, the amplification method of this locus needs further optimization.

3.1.6 Skin Community Analysis and Environmental Samples

One of the major challenges in the epidemiology of *Malassezia*-related diseases is the analysis of the (skin) microbial community structure in order to establish the role of *Malassezia* species in the various diseases, and to study the *Malassezia* community dynamics in time or as a consequence of treatment with antifungals. Multiple methods have been reported to identify *Malassezia* species from skin without culture of clinical material [71, 80, 185, 188, 190], but most of these methods require either separate amplification with specific primer sets or digestion of the amplification products (see RFLP above). Some real-time PCR protocols have been useful to study and quantify the presence of individual species (see PCR methods above). In microbial ecology, the study of community structure using the analysis of clone libraries obtained with taxon specific (e.g., panfungal) primers that target the rDNA locus has received considerable attention. This approach was applied to the analysis of psoriatic skin of five healthy humans and three patients with psoriasis [157]. The panfungal primers used targeted the SSU rDNA or were *Malassezia* specific based on the 5.8S rDNA and ITS 2 region (viz. Mal1F 5'-TCTTTGAACGCACCTTGC-3' and Mal1R 3'-CATGATACGTCATTTGCT-HT-5'). After cloning, the SSU rDNA amplicons were digested with HaeIII and the nucleotide order was determined [157]. The amplicons based on the Mal1F and Mal1R primers were also analyzed by digestion, in this case with DraI and XbaI, in order to specifically detect *M. globosa* and *M. restricta*. Amplicons that did not digest were sequenced to assess the species identity. Interestingly, this analysis yielded two unknown sequence types A and B (referred to as phylotypes A and B) that were the most prevalent sequences observed and that were closely related to, but not identical with *M. furfur.* On healthy skin, five *Malassezia* species were observed, with *M. restricta* dominating the samples from two subjects and *M. globosa* those from one other individual. *M. sympodialis, M. pachydermatis*, and *M. furfur* were observed as well. Healthy skin yielded four new phylotypes that may represent a yet undescribed species. Phylotype 1 occurred on all humans samples, whereas the other three phylotypes were found more rarely. In most of the healthy human beings, the *Malassezia* communities present on the right and left forearms were found to be similar. Three *Malassezia* species, namely *M. restricta, M. globosa*, and *M. sympodialis*, and an unknown phylotype occurred in psoriatic patients. Different samples obtained from one subject were similar, despite their site of isolation, from healthy or diseased skin, but those from the two other subjects showed differences in species composition. Similar to most other studies, and based on the number of clones detected, *M. restricta* and *M. globosa* were the most dominant species present in healthy and psoriatic skin [157].

In ecological studies, in which environmental samples were tested, DNA of *Malassezia* species has been detected in expected, but also in some unexpected habitats. Fourteen percent of 1,339 ITS clones made from indoor dust in Finland were related to various *Malassezia* species, and five out of 234 basidiomycetous sequences were related to *M. restricta* [164], thus indicating that *Malassezia* spp. may also occur abundantly in indoor

environments. DNA of *M. globosa* was detected in low moisture soil from Antarctic dry valley [68], DNA of *M. restricta* occurred in the gut of beetles originating from Southern Louisiana [211], as well as in nematodes from German soils [175], and *Malassezia* DNA was detected in methane hydrate-bearing deep-sea sediments [124]. These observations conflict with the supposed and, so far, exclusive occurrence of *Malassezia* yeasts on warm-blooded animals. It may well be that these observations imply that *Malassezia* yeasts occur in yet unknown environmental habitats. An alternative explanation may be that during sampling or processing of the environmental samples contamination by *Malassezia* DNA originating from other sources has occurred. Until the presence of *Malassezia* DNA and cells has been demonstrated unequivocally and undisputedly in such habitats, e.g., by using detection techniques combining both molecular and morphological characteristics, such as fluorescent *in situ* hybridization (FISH), their presence in such habitats remains putative.

3.1.7 Other Methods

Fourier transform infrared spectroscopy (FT-IRS) has been applied to identify *Malassezia* isolates [116]. This rapid method separated *M. furfur* from the remaining species. Isolates obtained from PV lesions were identified as *M. globosa* (62%), and those from dandruff as *M. furfur* (60%). As this latter observation conflicts with other published data, e.g., those from Gemmer et al. [80], FT-IRS needs further evaluation before its clinical application in *Malassezia* diagnostics.

3.2 Human Epidemiology

Takashi Sugita and Aristea Velegraki

3.2.1 Introduction

Although *Malassezia* species colonize the skin of healthy individuals, they cause pityriasis versicolor (PV), seborrheic dermatitis (SD), and *Malassezia* folliculitis (MF), and can exacerbate atopic dermatitis (AD). *Malassezia* species are also thought to be involved in psoriasis and acne vulgaris, although the detailed role of the microorganisms is unknown. *Malassezia* requires lipids for its growth; thus, it colonizes the sebaceous areas of the skin such as those on the face, scalp, and upper trunk, which are colonized to a greater extent than the limbs. It has also been shown that *Malassezia* species are heterogeneous. Although *M. furfur* has long been thought to cause or exacerbate several skin diseases, Guého et al. showed in 1996 [87] that *M. furfur* actually consists of five distinct species: *M. furfur*, *M. globosa*, *M. obtusa*, *M. restricta*, and *M. slooffiae* (see Chap. 2.1 for details). Since then, several studies were conducted, which aimed at elucidating the diseases caused by specific *Malassezia* species.

Initially, most of these studies involved qualitative comparisons of the *Malassezia* microbiota between lesion and nonlesion sites in patients, or between patients and healthy

individuals. More recently, quantitative analyses have been performed, as well as investigations into the relationships of the microbiota with aging or gender in healthy individuals, between children and adult patients, and among disease types. The *Malassezia* microbiota associated with different skin diseases are described in the following sections of this chapter. The apparent variability in the data may be explained by differences in race or region; it may also be the result of differences in sampling methods or culture media among the studies. Contact plates, swabs, and tape strips have been used to sample scale. Dixon media or Leeming and Notman agar (LNA) are used for *Malassezia* cultures. One member of the genus *Malassezia*, *M. restricta*, is often absent in cultures of clinical samples because its growth on plates is slower and therefore the species is easily overgrown by other isolated related species. All of the *Malassezia* species in a sample must be recovered from the medium to obtain an accurate quantitative analysis. Unfortunately, an optimized growth medium for all *Malassezia* species is not yet available. Culture-dependent methods can yield significant numbers of viable cells but have limited ability to produce accurate analytical results. Thus, methods that do not require an incubation period (i.e., molecular-based, culture-independent methods) have been developed to analyze the distribution of *Malassezia* microbiota.

In the molecular-based, culture-independent methods, sampling is performed using tape or a swab, and fungal DNA is subsequently extracted directly from the collected samples and analyzed by PCR. This method is not influenced by composition of the isolation medium, and thus slow-growing species, such as *M. restricta* and *M. obtusa*, may be detected. Even so, the results may be influenced by the DNA extraction method or the PCR detection sensitivity. In addition to *Malassezia*, fungi such as *Candida* and many bacteria colonize the skin. Therefore, high primer/probe specificity is needed to detect specific microbiota. In 2001, Sugita et al. introduced a DNA-based, culture-independent method for use in *Malassezia* research [188]. They used a transparent dressing to sample scale and nested PCR to increase the detection sensitivity for *Malassezia* DNA. In 2006, they also developed a quantitative real-time PCR method using TaqMan probes that allowed detection of as few as 10 copies of *Malassezia* DNA [193]. Retrospective studies also can be conducted using this procedure, as template DNA can be stored. This method is currently the most reliable and appropriate for analyzing *Malassezia* microbiota.

The number of accepted *Malassezia* species has doubled over the last decade, and many more species may exist. A comprehensive analysis using a clone library method is needed to identify all species of *Malassezia*. In this section, the epidemiology of each *Malassezia*-related skin disease and changes in the microbiota related to aging or gender in healthy individuals are described.

3.2.2 *Malassezia* in Healthy Individuals

The cutaneous *Malassezia* microbiota of an individual is formed immediately after birth. In a British study of 245 neonates (<28 days of age), skin swabs from 78 patients (31.8%) were positive for *Malassezia* on LNA, and 41 of 42 neonates were positive at follow-up [6]. In a second study examining 195 neonates in Iran [212], *M. furfur* and *M. globosa* were recovered from the medium in 60.5% and 7.2% of the patients, respectively; 31.3% of the cultures were negative for *Malassezia*. Recently, *M. dermatis* has been reported for the first time in 19 healthy individuals aged 17–55 years of age in Korea [125] by

culture-dependent methods. The identity of *M. dermatis* isolates was corroborated by sequencing of the ITS1 and 26S rDNA. However, quantitative studies to associate *M. dermatis* colonization and exacerbation or causation of skin disorders are pending.

Malassezia colonizes the sebaceous areas of the skin on the face, scalp, and upper trunk; the extent of colonization on the limbs is comparatively lower. Sebaceous gland activity is minimal in children, begins to increase at mid- to late childhood in response to androgen secretion, continues to increase until 16–19 years of age, and then shows no further significant change until old age [166]. Thus, given the dependency of sebaceous gland activity on age and gender, the *Malassezia* microbiota of healthy individuals should be assessed at every age and in both genders. Several studies, including two large-scale studies, have examined the *Malassezia* microbiota of healthy individuals. In 2004, Gupta et al. examined 245 healthy Canadian subjects [99]. The microorganisms were sampled by pressing contact plates filled with LNA to the skin, and positive colonies were identified based on their physiological features and molecular characteristics. The subjects were classified into groups (I–VI, respectively) by age: 0–3, 4–14, 15–25, 26–40, 41–60, and >60-years old. The rate of positive cultures in children ≤14 years of age was only 36%, whereas that in subjects ≥15 years of age was approximately 90%. The number of colonies recovered from the medium increased markedly beginning at age 15 (Fig. 3.4). The distributions of *M. furfur*, *M. globosa*, and *M. sympodialis* differed significantly among the age groups. In group I, *M. globosa* and *M. furfur* were the predominant species, accounting for 90% of the colonies; no *M. sympodialis* was found in group I. In groups II–VI, *M. globosa* and *M. sympodialis* accounted for 90% of the colonies. They found no significant difference between the genders in any age group.

In the second large-scale study, 770 healthy Japanese individuals aged 0–82 years were examined using a molecular-based, culture-independent method [194]. In males, the total amount of *Malassezia* DNA remained essentially constant between 0 and 9 years of age and then increased markedly up to 16–18 years of age (Fig. 3.5). In females, the amount of *Malassezia* DNA increased until 10–12 years of age, then decreased until 19–22 years of age,

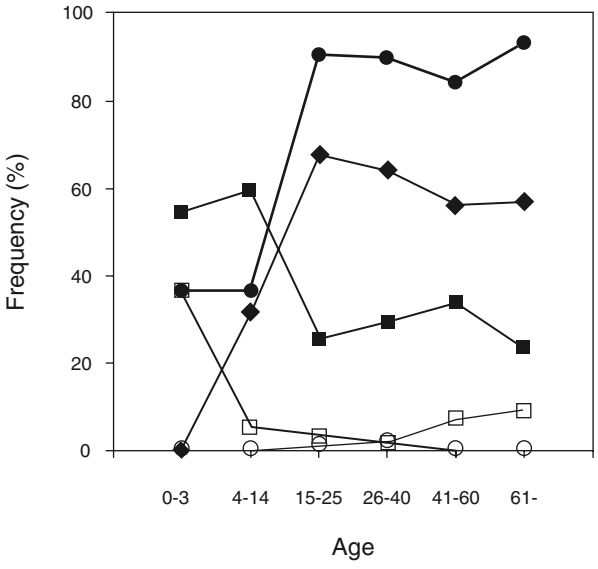

Fig. 3.4 Distribution of *Malassezia* species among individuals in different age groups as determined by culture-dependent analysis. The figure is based on original data from Gupta et al. [99]. Symbols indicate the number of individuals positive for *Malassezia* (closed circle), *M. sympodialis* (closed diamond), *M. globosa* (closed square), *M. furfur* (open square), and *M. restricta* (open circle)

and then increased again until 30–39 years of age before finally decreasing gradually with age beyond 39 years. Overall, males contained more *Malassezia* DNA than females. The ratio of total *Malassezia* colonization between males and females ranged from 0.7 to 3.4 until 15 years of age, after which it increased rapidly to 34 at 19–22 years of age (Fig. 3.5). *M. globosa* and *M. restricta* accounted for more than 70% of the total *Malassezia* DNA in both males and females at all ages. In males, *M. restricta* predominated at all ages and was overwhelmingly predominant after 16 years of age (Fig. 3.6). In females, *M. globosa* was dominant at 10–18 years of age, and *M. restricta* predominated over *M. globosa* after 23 years of age (Fig. 3.6).

Given that *Malassezia* requires lipids for growth, it is thought that changes in the *Malassezia* microbiota occur with changes in the composition of sebum, which consists of ceramides, fatty acids, cholesterol, cholesterol esters, squalene, triglycerides, and wax esters. Lipases secreted from cutaneous microorganisms hydrolyze the triglycerides into glycerin and free fatty acids, which serve as nutrients for the microorganisms. Yamamoto et al. [210] investigated the relationship between aging and the composition of saturated and unsaturated fatty acids (C_{14}–C_{18}), including straight and branched chains, in sebum. The proportion of C_{16} iso-branched fatty acids decreased markedly from infancy until the age of twenty and then increased slightly until senescence. The $C_{18:1}$ straight-chain component decreased during the twentieth year of age and did not change significantly thereafter. The percentages of $C_{15:1}$ and $C_{16:1}$ straight-chain components increased with age from infancy to the age of twenty and gradually decreased until the age of fifty. In contrast, the proportion of $C_{16:1}$ iso-branched fatty acids decreased from infancy until maturity, with its lowest point occurring at the age of twenty, and gradually increased until the age of fifty. Overall, the proportion of each fatty acid changed significantly at the age of twenty, as did the amount of sebum. Thus, age-related changes in the extent of colonization and the composition of the *Malassezia* microbiota are closely associated with age-related changes in sebaceous gland activity and the fatty acid composition of sebum. In addition, cosmetics may have an effect on the microbiota, as preservatives included in the cosmetics can inhibit the growth of *Malassezia*.

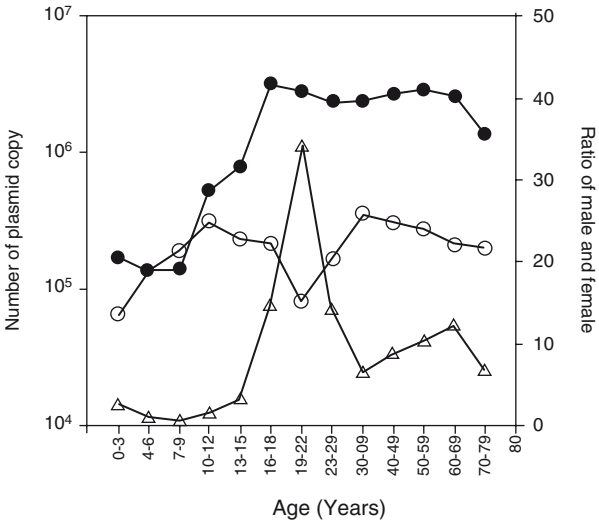

Fig. 3.5 Change in the amount of *Malassezia* DNA present in humans of different age. Males (closed circle), females (open circle), ratio of the amount of *Malassezia* DNA in males vs. females (open triangle)

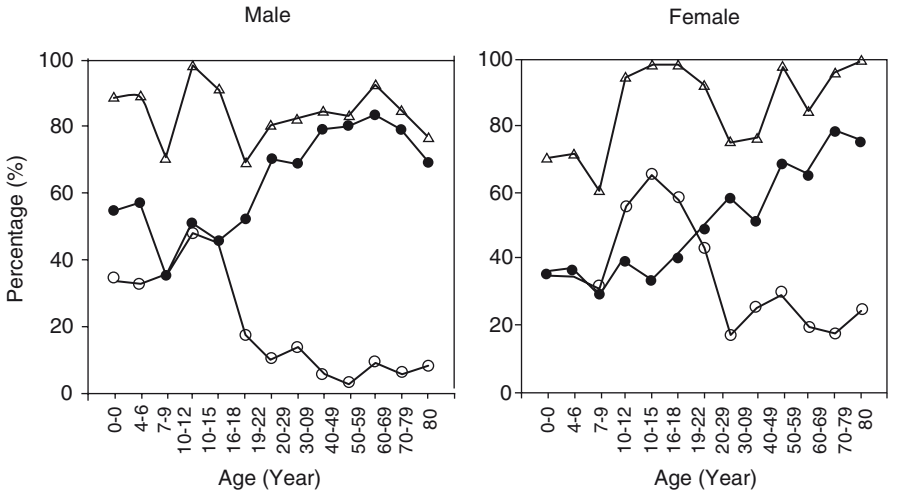

Fig. 3.6 Proportions of the predominant species *Malassezia globosa* and *M. restricta* in males and females, respectively. *M. globosa* (closed circle), *M. restricta* (open circle), *M. globosa* plus *M. restricta* (triangle)

In summary, the *Malassezia* microbiota of healthy individuals is gender-dependent and changes with age. Nevertheless, *M. globosa* and *M. restricta* predominate in both genders and in all age groups.

3.2.3 *Malassezia*-Related Skin Diseases

3.2.3.1 Pityriasis Versicolor

Pityriasis versicolor (PV) is a superficial infection of the stratum corneum caused by *Malassezia*. It is most commonly found on the trunk and upper arms of people in their twenties and thirties, occurring often during the summer but not during the winter. Direct microscopic examinations using methylene blue stain have revealed hyphae and yeast cells among the scales collected from the lesions of PV patients; however, hyphae are rare in other *Malassezia*-related diseases.

Studies of the *Malassezia* microbiota in PV have been conducted in several counties; six representative studies are summarized in Table 3.8. *M. globosa* was isolated at a rate of 60% by culture-dependent analysis in a Spanish study (Southwest Europe) [53] (Table 3.8). A Greek study (Southeast Europe) also suggested that *M. globosa* was a major *Malassezia* species in PV patients [73]; *M. globosa* was identified alone or in combination with other *Malassezia* species in 77 or 12.7% of the *Malassezia*-positive then patients, respectively. In a Canadian study, *M. sympodialis* (68.8%) was the predominant species, followed by *M. furfur* (12.3%) and *M. globosa* (6.3%) [101]. Also, in studies conducted in Iran (southwest

Table 3.8 Distribution of *Malassezia* species in patients with pityriasis versicolor

Analytical methods	Culture-dependent methods					Culture-independent method
Countries References	Spain [53]	Canada [101]	Iran [198]	Greece [73]	Bosnia and H* [171]	Japan [141]
Number of patients	96	23	94	71	90	49
M. globosa[a]	60[b]	6[b]	53[b]	77[b]	63[b]	94[b]
M. restricta	NI	NI	NI	2[c]	NI	94[b]
M. sympodialis	33[c]	69[b]	9[b]	13[c]	14[b]	35[b]
M. dermatis	NI	NI	NI	NI	NI	24[b]
M. furfur	NI	12[b]	25[b]	5[c]	10[b]	10[b]
M. obtusa	NI	6[b]	8[b]	NI	8[b]	8[b]
M. japonica	NI	NI	NI	NI	NI	6[b]
M. slooffiae	7[c]	6[b]	4[b]	3[c]	5[b]	4[b]
M. yamatoensis	NI	NI	NI	NI	NI	4[b]
Medium used for isolation	mDA	LNA	DA	mDA	mDA	–

DA Dixon agar; *mDA* modified Dixon agar; *LNA* Leeming and Notman agar; *NI* not identified; *H** Herzegovina

[a]Number indicates the percentage (%) of the total number of patients with PV

[b]Samples collected from lesional sites

[c]Percentage refers to co-isolation with *M. globosa*

Asia) and in Bosnia and Herzegovina (the Balkan peninsula) *M. globosa* was the predominant species isolated from lesional sites [171, 198, 213] followed by *M. sympodialis* and *M. furfur*. Little or no *M. restricta* (Table 3.8) was isolated from patients in these studies using culture-dependent analysis. However, *M. globosa* and *M. restricta* were detected in almost all PV patients by nested PCR using species-specific primers in a Japanese study [141]. Real-time PCR revealed that *M. globosa* DNA accounted for 62.8±18.1% of all *Malassezia* DNA (Fig. 3.2), and the extent of colonization of *M. globosa* was six times that of *M. restricta* [184]. The detection frequency of *M. sympodialis* was 34.6%, and that of the other species ranged from 4.1 to 24.4%. Thus, DNA-based analyses suggest that *M. globosa* is qualitatively and quantitatively the predominant species in the scale of PV patients.

Hyphae are frequently observed in the scale of PV patients; therefore, the presence of hyphae is important in elucidating the causative agent of PV. Following treatment with antifungal agents, yeast cells were detected in the scale of PV patients, whereas hyphae were not observed, in accordance with the reported improvement of the patients' symptoms. Only *M. globosa* DNA was detected by PCR when scale samples containing only hyphae were harvested [141]. Furthermore, no report has shown that *M. restricta* develops hyphae in vitro. Taken together, these data suggest that *M. globosa* plays a significant role in the pathogenesis of PV.

3.2.3.2 Seborrheic Dermatitis

Seborrheic dermatitis (SD) is a common inflammatory skin disease associated with seborrhea. It occurs most often on areas of the body that are rich in sebaceous glands, such as the face, scalp, and upper trunk. The disorder is present in 1–3% of the general population and is more prevalent in men than in women; it is frequently observed in patients with acquired immunodeficiency syndrome (AIDS), with an incidence of 30–33% [8, 98]. SD is also observed in patients with Parkinson's disease. In addition, SD is among the new secondary effects appearing recently with the use of antitumor agents, such as cetuximab and erlotinib, an epidermal growth factor (EGF) receptor inhibitor [176]. The condition often has a seasonal aspect, being more common and, in chronic cases, more severe in the winter months. The cause of SD has not been clarified; however, several possible contributors to the development of the disorder, including exogenous and endogenous factors, have been described. Of these, *Malassezia* has generated a large body of data, indicating that it plays a major etiological role in SD. The clinical condition is improved in accordance with a reduction in the number of *Malassezia* cells in the lesions following antifungal treatment with itraconazole or ketoconazole, suggesting that *Malassezia* is strongly associated with the development of SD [102]. The free fatty acids produced by the hydrolysis of sebum by lipases serve as nutrients for cutaneous microorganisms and also induce inflammation of the skin.

The distribution of *Malassezia* in patients with SD from various countries is summarized in Table 3.9. A culture-dependent analysis from Canada showed that *M. globosa* was the predominant species (45.0%) in lesions, followed by *M. sympodialis* (30.8%) and *M. slooffiae* (10%) [101]. Moreover, the trunk was found to be the most highly colonized site on the body, and the overall recovery rate was 82.1%. However, the recovery rate in studies from Sweden and Japan was much lower. *M. globosa*, *M. furfur*, and *M. sympodialis* were recovered in 20.8, 20.8, and 6.3% of the samples, respectively, in a Japanese study [145], and *M. sympodialis*, *M. obtusa*, and *M. slooffiae* were recovered in 25, 25, and 12.5% of the samples, respectively, in a Swedish study [180]. A common feature among these three studies is that no *M. restricta* was recovered from the samples. In a Greek study of SD patients, *M. globosa* was isolated alone (33.3%), in combination with either *M. sympodialis* or *M. restricta* (13.3%), and together with both *M. sympodialis* and *M. restricta* (1 patient 2.2%). It was concluded in that study that *M. globosa* was the prevalent species in SD patients and occurred either alone or in combination with other *Malassezia* species [73]. However, by culture-independent analysis, a Japanese study showed that *M. globosa* and *M. restricta* were the predominant species at both lesion and nonlesion sites in SD, accounting for 5.2 ± 7.9% and 72.3 ± 15.0%, respectively, of the *Malassezia* DNA detected by real-time PCR [195] (Fig. 3.2). *Malassezia sympodialis*, *M. dermatis*, and *M. slooffiae* were present at rates of 25.8–35.5% at lesion sites and at rates of 14.8–22.2% at nonlesion sites; other species, including *M. furfur*, *M. obtusa*, and *M. yamatoensis*, were detected at rates below 10% at lesion and nonlesion sites.

As for colonization by *Malassezia* cells, the results varied as well. One study indicated a correlation between the density of *Malassezia* cells and the clinical severity of SD [111], yet another study indicated that the number of *Malassezia* colonies at lesion sites was significantly lower than that at nonlesion sites [101]. A DNA-based study revealed that the *Malassezia* microbiota at lesion sites was more diverse than at nonlesion sites [195], that

Table 3.9 Distribution of *Malassezia* species in patients with seborrhoeic dermatitis

Analytical methods	Culture-dependent methods				Culture-independent methods			
Countries References	Canada [101]	Japan [145]	Sweden [180]	Greece [73]	Japan [195]		USA [80]	
Number of patients	28	48	16	38	31	46	24	
M. globosa[a]	45[b]	21[b]	6[b]	58[b,c]	93[b]	70[d]	46[e]	33[f]
M. restricta	38	NI	NI	48[b,c]	61[b]	56[d]	72[e]	50[f]
M. sympodialis	37[b]	6[b]	25[b]	8[b,c]	26[b]	15[d]	7[e]	8[f]
M. dermatis	8[b]	NI	NI	NI	35[b]	19[d]	NI	NI
M. furfur	NI	21[b]	6[b]	2[b,c]	6[b]	NI	NI	NI
M. obtusa	NI	NI	25[b]	NI	10[b]	NI	2[e]	NI
M. japonica	NI	NI	13[b]	NI	13[b]	4[d]	NI	NI
M. slooffiae	10[b]	NI	12[b]	5[b,c]	32[b]	22[d]	4[e]	NI
M. yamatoensis	NI	NI	NI	NI	10[b]	4[d]	4[e]	NI
Medium used for isolation	LNA	DA	LNA	mDA	–	–	–	–

DA Dixon agar; *mDA* modified Dixon agar; *LNA* Leeming and Notman agar; *NI* not identified
[a]Number indicates the percentage for total number of patients
[b]Samples collected from lesional sites
[c]Co-isolation of mixed *Malassezia* species from lesions
[d]Samples collected from nonlesional sites
[e]Samples collected from patients at >24 of adherent scalp flaking scale (ASFS)
[f]Samples collected from patients at <10 of ASFS

the detection frequency of each species in lesion skin was higher than that in nonlesion skin, that the average numbers of species per individual in lesion and nonlesion skin were 2.8 ± 1.6 and 1.9 ± 1.2, respectively, and that the overall colonization by *Malassezia* was higher in lesion skin than in nonlesion skin.

Dandruff is an exceptionally common scalp condition characterized by flaking and scaling. Dandruff can be thought of as either a mild variant of SD or as a separate condition that is capable of worsening into true SD. The key distinction between dandruff and SD is that there is no visible inflammation associated with dandruff. Gemmer et al. [80] first attempted to detect *Malassezia* from dandruff samples using a DNA-based approach (see Sect. 3.1.4.4). The subjects were separated into two groups (<10 and >24) based on scoring according to an adherent scalp flaking scale (ASFS). Both *M. globosa* and *M. restricta* were predominant in the dandruff from each group. Those subjects with a high composite ASFS score were more likely to be positive for *Malassezia* (*M. restricta*, 71.7% for a high composite score vs. 50.0% for a low composite score; *M. globosa*, 45.7% for a high composite score vs. 33.3% for a low composite score). *M. sympodialis*, *M. slooffiae*, *M. obtusa*, and *M. furfur* were detected in a very small percentage of the subjects from each group. A significant percentage of subjects in each group (28.3% with ASFS score <10; 28% with

ASFS score >24) were positive for non-*Malassezia* species. To date, no quantitative analysis of dandruff has been reported.

3.2.3.3 Atopic Dermatitis

Atopic dermatitis (AD), which is defined as dermatitis with pruritus, is a chronic disease involving cycles of remission and deterioration. AD is characterized by hypersensitivity to dry skin, which may involve IgE antibodies and various environmental factors. As the barrier function of the cutaneous surface is low in AD patients, their skin is easily stimulated by normal cutaneous microorganisms. A relationship between AD and cutaneous *Malassezia* has been suggested based on the fact that anti-*Malassezia* IgE antibodies have been detected in patients with AD but not in healthy subjects. In particular, in head and neck AD, there is a significant correlation between the level of anti-*Malassezia* IgE and clinical severity [6]. In addition, the symptoms of AD patients may be improved by antifungal agents such as ketoconazole and itraconazole, according to a reduction in the number of *Malassezia* colonies in their lesions [55, 151]. Currently, *Malassezia* is considered to be an exacerbating factor in AD.

A study conducted by Nakabayashi et al. [145] detected *M. furfur*, *M. globosa*, *M. sympodialis*, and *M. slooffiae* in 21.4, 14.3, 7.1, and 3.6% of samples from Japanese AD patients, respectively (Table 3.10). A study performed in Sweden [180] produced similar data, while a Canadian study by Gupta et al. [101] showed that *M. sympodialis* was the predominant species in patients with AD (detection rate, 51.3%). Common among the three culture-dependent studies is that *M. restricta* was rarely or never isolated from the medium (0–7.7%). With the detection of *Malassezia* by a molecular-based, culture-independent method, which may be considered to be superior for the detection of *Malassezia* spp. by traditional methods, Tajima et al. [195] found both *M. globosa* and *M. restricta* to be the predominant species in AD, while the detection rate of *M. sympodialis* was 58.3% and the detection rates for other species ranged from 13.9 to 33.3%. The serum levels of IgE antibodies against the two major species were also found to be higher than those against other species [118].

The onset of AD usually occurs during the first 6 months of life, and the prevalence of AD, which is similar in the United States, Europe, and Japan, is increasing. AD is classified into three sequential phases: infantile, childhood, and adult. Although the incidence of AD decreases with age, the severity of AD tends to be greater in adults. The species of the *Malassezia* microbiota were similar between children with AD (9.7 ± 1.5-years old) and adults with AD (36.1 ± 9.5-years old). In addition, *M. globosa* and *M. restricta* were detected in 100% of child and adult AD patients, whereas the other species were detected in no more than 40% of the cases. Totals of 3.2 ± 1.3 and 2.8 ± 1.1 species were detected in samples from children and adults with AD, respectively [197]. Quantitatively, the *Malassezia* microbiota differed significantly between children and adults with AD. Colonization by *Malassezia* in adults was 2.4-fold of that in children. The percentages of *M. globosa* and *M. restricta* were similar in adults, while *M. restricta* was the predominant species in children. The serum level of *Malassezia*-specific IgE antibodies was lower in

Table 3.10 Distribution of *Malassezia* species in patients with atopic dermatitis

Analytical methods	Culture-dependent methods			Culture-independent methods
Countries References	Japan [145]	Canada [101]	Sweden [180]	Japan [195]
Number of patients	28	31	124	31
M. globosa[a]	14[b]	18[b,c]	11[b]	100[b]
M. restricta	NI	8[b,c]	3[b]	97[b]
M. sympodialis	7[b]	51[b,c]	NI	58[b]
M. dermatis	NI	NI	NI	31[b]
M. furfur	21[b]	10[b,c]	NI	33[b].
M. obtusa	NI	10[b,c]	5[b]	28[b]
M. japonica	NI	NI	NI	33[b]
M. slooffiae	4[b]	3[b,c]	3[b]	31[b]
M. yamatoensis	NI	NI	NI	14[b]
Medium used for isolation	DA	LNA	LNA	–

DA Dixon agar; *mDA* modified Dixon agar; *LNA* Leeming and Notman agar; *NI* not identified
[a]Number indicates the percentage (%) for total number of patients
[b]Samples collected from lesional sites
[c]Samples collected from nonlesional sites

children than in adults. Antifungal agents have been given as an alternative therapy to AD patients; however, clinical improvement of the patients' symptoms has been observed only in adults. It appears that antifungal therapy (e.g., ketoconazole or itraconazole) may be effective in adult cases of AD affecting the head and neck [55].

3.2.3.4 *Malassezia* Folliculitis

Malassezia folliculitis (MF) involves pruritic papules and pustules that occur mainly on the trunk and upper arms and may also be seen in association with pregnancy, leukemia, and Hodgkin's disease in immunocompromised patients [6]. The development of MF may be related to temperature and humidity, as MF appears to be more common in tropical countries.

Follicular occlusion is a primary event in the development of folliculitis, with *Malassezia* overgrowth as a secondary occurrence. Propionibacteria and staphylococci are found distributed within affected follicles, although few studies of MF have attempted to culture the follicular contents. A recent culture-dependent study of 32 Japanese patients demonstrated that the predominant species recovered from folliculitic lesions were *M. globosa* and *M. sympodialis*, while *M. restricta*, *M. globosa*, and *M. sympodialis* were isolated using culture-independent methods [4].

3.2.3.5 Psoriasis

Psoriasis (PS) is a multifactorial immune skin disease whose etiology involves microbial environmental factors in combination with a strong genetic component, including several genes encoding proteins involved in epidermal differentiation and immune, inflammatory, and pathogen responses. Various microorganisms are considered to be associated with the provocation and exacerbation of PS; these include *Streptococcus pyogenes*, *Staphylococcus aureus*, *Malassezia* spp., and *Candida albicans* [70]. The association between PS and *S. pyogenes* was first noted 50 years ago. However, our knowledge of *Malassezia* and PS is not as extensive as our knowledge of PV, SD, and AD. *Malassezia* upregulates keratinocyte expression of transforming growth factor-beta1 and heat shock protein 70, which are associated with hyperproliferation and cell migration in the epidermis and which are more highly expressed in psoriatic skin colonized with *Malassezia* than in noncolonized psoriatic skin [70]. Antifungal treatment has been shown to improve psoriatic scalp lesions [67]. In a Canadian study using culture-dependent methods, *M. globosa* (57.7%) and *M. sympodialis* (30.8%) were found to be the predominant species in the lesions of patients with PS (Table 3.11) [101]. In 40 psoriatic patients with scalp involvement studied in Bosnia and Herzegovina [170] on the Balkan Peninsula, *M. globosa* was also the predominant (55%) lesional isolate, followed by *M. slooffiae* (17.5%) and *M. restricta* (10%) but, surprisingly, *M. sympodialis* was absent. In that culture-based study, *M. restricta* was the predominant isolate form healthy scalp specimens of 40 clinically healthy individuals and significant difference in the distribution of *Malassezia* species was recorded between psoriatic and healthy scalp. An Iranian study of 110 PS patients and 123 healthy individuals reported that the frequency of positive cultures among the PS patients was lower than that among the healthy controls (62.7 vs. 87.0%, respectively) [213]. *Malassezia globosa*, *M. furfur*, and *M. restricta* were predominantly recovered in psoriatic scalp cultures (Table 3.11).

Using a PCR-based, culture-independent method, Amaya et al. [5] showed that *M. globosa* (68.2%) and *M. restricta* (90.9%) were the major components, followed by *M. sympodialis* (50.0%), in the lesions of 22 PS patients (Table 3.11). Colonization by *M. restricta* was five times the colonization by *M. globosa* at all body sites. In addition, *Malassezia* colonization was significantly lower in patients with hyperlipidemia than in patients with normolipidemia [196]. No correlation has been demonstrated between the psoriasis area and severity index (PASI) and the amount of colonization by *Malassezia*.

A comprehensive analysis of PS patients using a clone library was performed by Paulino et al. [157], who analyzed 25 skin samples from five healthy subjects and three PS patients using broad-range 18S rDNA and 5.8S rDNA/ITS2 *Malassezia*-specific primers. An analysis of 1,374 clones identified five species (*M. globosa*, *M. restricta*, *M. sympodialis*, *M. furfur*, and *M. pachydermatis*) and four unknown phylotypes, potentially representing new species. The *Malassezia* microbiota did not change significantly over 6 months. Samples of the *Malassezia* microbiota from healthy skin and psoriatic lesions were similar in one patient but substantially different in two others. No consistent pattern differentiating psoriatic skin from healthy skin was found.

Table 3.11 Distribution of *Malassezia* species in patients with psoriasis

Analytical methods	Culture-dependent methods			Culture-independent methods	
Countries References	Canada [101]	Bosnia and H* [170]	Iran [213]	Japan [5]	Japan [196]
Number of patients	28	40*	110	22	20
M. globosa[a]	58[b]	55[b]	47[c,d]	68[b]	98[b]
M. restricta	NI	10[b]	11[d]	91[b]	92[b]
M. sympodialis	31[b]	2[b]	3[d]	50[b]	NI
M. dermatis	NI	NI	NI	14[b]	NI
M. furfur	NI	8[b]	39[d]	18[b]	NI
M. obtusa	NI	NI	NI	18[b]	NI
M. japonica	NI	NI	NI	13[b]	NI
M. slooffiae	11[b]	17[b]	NI	14[b]	NI
M. yamatoensis	NI	NI	NI	10[b]	NI
Medium used for isolation[e]	LNA	mDA	LNA	–	–

mDA modified Dixon agar; *LNA* Leeming and Notman agar; *NI* not identified; *H** Herzegovina
*Three out of forty patients with negative cultures
[a]Number indicates the percentage (%) for total number of patients
[b]Samples collected from lesional site
[c]Samples collected from scalp
[d]Percentage refers to co-isolation with other *Malassezia* species

3.2.3.6 Genotype analysis of the two predominant species, *M. globosa* and *M. restricta*

In the rRNA gene, the IGS region, which is located between the small and large subunits, shows a remarkable diversity in fungi. The IGS region is further divided into two subregions, IGS1 and IGS2. A phylogenetic tree constructed from the IGS1 sequences of *M. globosa* consisted of eight clusters (Fig. 3.7), each of which corresponded to a different subject or patient group (i.e., healthy subjects, patients with AD, and patients with SD) [190, 195]. Cluster VII corresponded to the sequences derived from healthy subjects only, whereas clusters I and VIII corresponded to sequences from both AD/SD patients and healthy subjects. The remaining five clusters were specific to patients. *M. globosa* IGS1 has four short sequence repeats (SSRs): one $(GT)_n$ and three $(CT)_n$. The number of (CT) repeats in the $(CT)_n$ SSRs was more variable in the healthy subjects than in the AD patients, who exhibited 4–11 (CT) repeats at CT1; 3–10, at CT2; and 3–11, at CT3. This corresponds to four (CT) repeats in 50% of the samples from AD patients, eight repeats in 60%, and 9–11 repeats in 80%. For the $(GT)_n$ SSR of IGS1, the numbers of (GT) repeats in 70–80% of the samples from AD patients and healthy subjects were 9–11 and 15–19, respectively. For the *M. restricta* IGS1 sequences, the tree consisted of two major clusters: cluster I corresponding to patients and healthy subjects, and cluster II corresponding to patients only. Genotypic

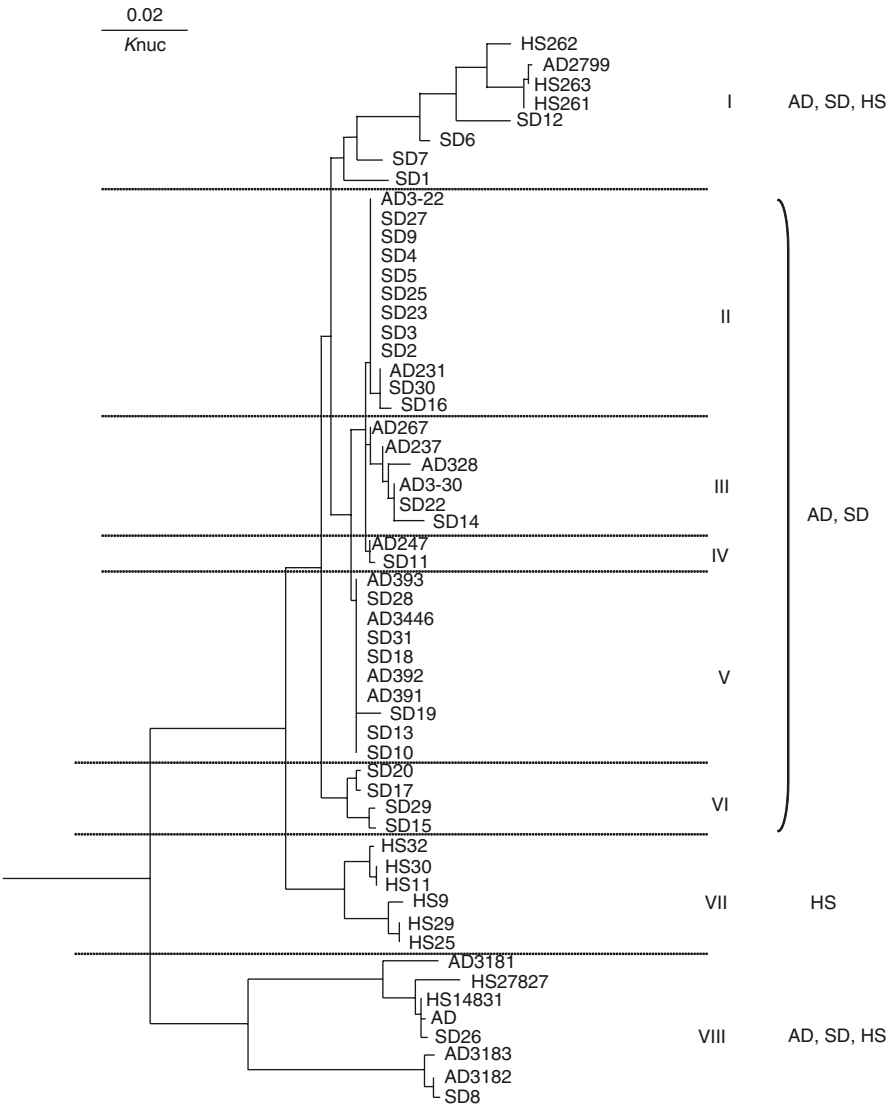

Fig. 3.7 Phylogenetic tree of *M. globosa* based on its IGS sequence. AD atopic dermatis; SD seborrheic dermatitis; HS healthy subject.

analysis of the IGS1 sequences could not separate AD patient-derived *M. globosa*/*M. restricta* from SD patient-derived *M. globosa*/*M. restricta*. Gaitanis et al. [73] reported that sequences of *M. globosa* isolated from PV patients were divided into five groups by single-strand conformational polymorphism analysis of the ITS1 region. One genotype of *M. globosa* appeared to be associated with extensive clinical disease.

3.3 Animal epidemiology

Jacques Guillot, Suzana Hađina and F. Javier Cabañes

3.3.1 Introduction

Malassezia yeasts belong to normal cutaneous or mucosal microbiota of many warm-blooded vertebrates. These yeasts are now recognized as opportunistic pathogens that play a significant role in the development of different animal diseases such as otitis externa (OE) or seborrhoeic dermatitis (SD).

Malassezia species have an affinity for lipids as substrates and the term "lipophilic yeasts" is frequently used to characterize the genus. In fact, most of the species show an absolute requirement for long fatty acid chains and they are, therefore, rarely isolated in the laboratory unless specific nutrients are provided in the medium. In 2006, lipid-dependent yeasts included ten species: *M. sympodialis, M. globosa, M. restricta*, *M. furfur, M. obtusa, M. slooffiae, M. dermatis, M. japonica*, *M. yamatoensis*, and *M. nana.* Of these, three species (*M. dermatis, M. japonica*, and *M. yamatoensis*) have so far been isolated exclusively from human skin. Recently, two new species that inhabit animals have been described: *M. equina* from healthy skin of the anus from horses, and *M. caprae* from the ear skin of healthy goat [31]. *M. pachydermatis* is the only non lipid-dependent species; it grows on Sabouraud glucose agar without any special additional requirements.

Epidemiology is traditionally defined as the study of the occurrence, distribution, and control of diseases in populations. In the present chapter, the occurrence and distribution of *Malassezia* yeasts among animal hosts will be described. The second part of this chapter is dedicated to the description of predisposing factors leading to *Malassezia* overgrowth on the skin of animals.

3.3.2 Occurrence and Distribution of *Malassezia* Yeasts in Animals

Malassezia yeasts have been isolated from almost all domestic animals, different wild animals held in captivity, but also from wildlife. Tables 3.12 and 3.13 show the animal species from which *Malassezia* yeasts have been isolated.

3.3.2.1 Animals in Zoological Parks or in Wildlife

A few epidemiological studies aimed at detecting the presence of *Malassezia* yeasts from the skin of different animal species. In 1925, Weidman was the first to observe *Malassezia* yeasts from an animal [204]. He described bottle-shaped yeast-like cells in scales from an Indian rhinoceros (*Rhinoceros unicornis*) with exfoliative dermatitis (Fig. 3.8). This microorganism

Table 3.12 *Malassezia* yeasts isolated from wild animals

Animals	Non lipid dependent species (*M. pachydermatis*) isolated from					Lipid-dependent yeasts[a] isolated from					References
	A	B	C	D	E	A	B	C	D	E	
Bear (different species)	+	+	–	–	–	–	–	–	-	–	[178]
	+	–	–	+	–	–	–	–	–	–	[107]
	+	–	–	–	–	–	–	–	–	–	[91, 120, 123]
Wolf (*Canis lupus*)	+	–	–	–	–	–	–	–	–	–	[107]
Coyote (*Canis latrans*)	–	+	–	–	–	–	–	–	–	–	[179]
Fox (*Vulpes vulpes*)	–	+	–	–	–	–	–	–	–	–	[179]
	+	–	–	+	–	–	–	–	–	–	[107]
	+	–	–	–	–	–	–	–	–	–	[54]
Fennec fox (*Vulpes zerda*)	+	–	–	–	–	–	–	–	–	–	[91]
Ferret (*Mustela putorius*)	+	–	–	–	–	–	–	–	–	–	[58, 91]
Large felids (different species)	–	–	–	–	–	–	2	–	–	–	[91]
	–	–	–	–	–	2	–	–	–	–	[47]
Small felids (different species)	+	–	–	–	–	–	–	–	–	–	[47]
Gray seal (*Halichoerus gripus*)	+	–	–	–	–	–	–	–	–	–	[91]
Sea lion *(Zalophus californianus)* (*Otaria byronia*)	+	+	+	–	–	–	–	–	–	–	[94]
	–	+	–	–	–	–	–	–	–	–	[146]
Porcupine (*Erethizon dorsatum*)	–	+	–	–	–	–	–	–	–	–	[179]
Badger (*Meles meles*)	+	–	–	–	–	–	–	–	–	–	[123]
Bat (*Molossus molossus*)	+	–	–	–	–	2,3,4	–	–	–	–	[77]
Big anteater (*M. tridactyla*)	+	–	–	–	–	–	–	–	–	–	[123]
Doe (*Capreolus capreolus*)	–	+	–	–	–	–	–	–	–	–	[106]
Camel (*Camelus dromedarius)*	–	–	–	–	–	–	–	–	–	–	[122]
Okapia (*Okapia johnstoni*)	–	–	–	–	–	1	–	–	–	–	[91]
Rhinoceros (*Rh. unicornis)* (*C. simum simum)*	+	–	–	–	–	–	–	–	–	–	[123]
	–	+	–	–	–	–	–	–	–	–	[9, 204, 205]
(*D. s. bicornis michaeli*)	+	+	–	–	–	1	–	–	–	–	[91]
Elephant (*Elephas maximus)*	+	+	–	–	–	1	1	–	–	–	[91]
	+	–	–	–	–	–	–	–	–	–	[123]
Wombat (*Vombatus ursinus*)	+	–	–	–	–	–	–	–	–	–	[123]
	–	+	–	–	–	–	–	–	–	–	[91]
Non human primates (different species)	–	–	–	–	–	–	1	–	–	–	[137]
	+	+	–	–	–	–	1	–	–	–	[91]
Birds (different species)	–	+	–	–	–	–	–	–	–	–	[66]
	–	+	–	–	–	–	1	–	–	–	[89, 137, 177]
	–	–	+	–	–	–	–	–	–	–	[29]

Localization of sampling is indicated with a capital letter: *A* external ear canal; *B* body skin (lips, axilla, groin, dorsum, pinna, precrural area, ventral midline, udder), mucosa, hair, nails, fur, feathers, and comb; *C* mouth; *D* perianal area, anus; *E* eye, conjunctival sac

[a]Key for lipid-dependent yeasts: *1* not identified or not specified; *2 M. sympodialis*; *3 M. globosa*; *4 M. furfur*

Table 3.13 *Malassezia* yeasts isolated from domestic animals

Animals	Non lipid-dependent yeasts (*M. pachydermatis*)					Lipid-dependent yeasts[a] isolated from					References
	A	B	C	D	E	A	B	C	D	E	
Dog (*Canis familiaris*)	+	–	–	–	–	–	–	–	–	–	[33, 81, 104, 121, 128, 133, 152]
	+	+	–	–	–	–	–	–	–	–	[88, 91, 95, 120, 132, 174, 182]
	–	+	–	–	–	–	–	–	–	–	[20, 21, 24, 27, 28, 36, 66, 119, 154]
	+	+	–	+	–	–	–	–	–	–	[17]
	+	+	+	+	–	–	–	–	–	–	[22, 108]
	+	+	–	+	–	1	1	–	1	–	[34]
	+	+	+	+	+	–	–	–	–	–	[127, 148]
	–	+	+	+	–	–	–	–	–	–	[18]
	+	+	–	–	+	–	–	–	–	–	[168]
	+	–	–	+	–	–	–	–	–	–	[40]
	–	–	–	–	–	4	–	–	–	–	[63]
	+	–	–	–	–	4, 6	–	–	–	–	[50]
	+	–	+	+	–	–	–	–	–	–	[162]
	–	+	+	–	–	–	–	–	–	–	[19]
	–	–	–	–	+	–	–	–	–		[167]
Cat (*Felis catus*)	+	+	–	–	–	–	–	–	–	–	[10, 40, 88, 150, 174, 182]
	+	+	–	+	–	2	2, 3	–	–	–	[25]
	+	–	–	–	–	2	–	–	–	–	[59]
	+	–	–	–	–	2, 3, 4	–	–	–	–	[147]
	+	–	–	–	–	3	–	–	–	–	[33]
	–	+	–	–	–	–	–	–	–	–	[66]
	–	–	–	–	–	2	–	–	–	–	[49]
	–	–	–	–	–	8	–	–	–	–	[112, 113]
	+	–	–	–	–	4	–	–	–	–	[48]
	–	+	–	–	–	–	2, 4	–	–	–	[45]
	–	–	–	+	–	–	–	–	–	–	[109]
	+	+	–	+	–	–	5	–	–	–	[1]
	+	+	+	+	–	8	5, 8	–	5	–	[159]
	–	–	–	+	–	2	2	–	2	–	[23]
	–	+	–	–	–	–	1	–	–	–	[155]
Swine *(Sus scrofa)*	–	–	–	–	–	1	–	–	–	–	[106, 122]
	–	–	–	–	–	–	1	–	–	–	[105]
	–	+	–	–	–	–	–	–	–	–	[66]
	+	–	–	–	–	–	–	–	–	–	[40, 123, 163]
	–	–	–	–	–	2, 5	–	–	–	–	[78]
	–	–	–	–	–	1	–	–	–	–	[91]

Table 3.13 (continued)

Animals	Non lipid-dependent yeasts (*M. pachydermatis*)					Lipid-dependent yeasts[a] isolated from					References
	A	B	C	D	E	A	B	C	D	E	
Horse *(Equus caballus)*	–	–	–	–	–	1	–	–	–	–	[106, 183]
	–	+	–	–	–	–	–	–	–	–	[66]
	–	–	–	–	–	–	2	–	–	–	[182]
	+	+	–	+	–	4,5,6,7	2,3,4,5,7	–	3,4,5,6	–	[182]
	–	–	–	–	–	–	1	–	1	–	[149]
	–	+	–	+	–	–	–	–	–	–	[40]
	–	–	–	–	–	–	5, 9	–	–	–	[207]
	–	–	–	–	–	–	–	–	9	–	[31]
Bovine *(Bos taurus)*	–	+	–	–	–	–	–	–	–	–	[66]
	–	–	–	–	–	–	1	–	–	–	[106]
	+	–	–	–	–	2,3,4,5,6	–	–	–	–	[61, 63]
	–	–	–	–	–	2, 4	–	–	–	–	[62]
	–	–	–	–	–	3, 4	2, 4, 7	–	–	–	[51]
	1	–	–	–	–	1	–	–	–	–	[65]
	–	–	–	–	–	8	–	–	–	–	[113]
	–	–	–	–	–	–	5	–	–	–	[91]
Goat *(Capra hircus)*	–	–	–	–	–	5	1	–	–	–	[89]
	+	–	–	–	–	2, 6	3, 7	–	4	–	[51]
	–	+	–	–	–	–	–	–	–	–	[161]
	+	–	–	–	–	–	–	–	–	–	[40]
	–	–	–	–	–	–	5	–	–	–	[200]
	–	–	–	–	–	–	1	–	–	–	[12]
	–	–	–	–	–	–	10	–	–	–	[31]
Sheep *(Ovis aries)*	–	–	–	–	–	–	1	–	–	–	[91]
	–	–	–	–	–	–	2, 3, 7		–	–	[51]
Chicken *(Gallus gallus)*	–	–	–	–	–	–	2	–	–	–	[84]
Guinea pig *(Cavia porcellus)*	–	+	–	–	–	–	–	–	–	–	[66]
Rabbit *(Oryctolagus cuniculi)*	–	+	–	–	–	–	–	–	–	–	[66]

Localization of sampling is indicated with a capital letter: *A* external ear canal; *B* body skin (lips, axilla, groin, dorsum, pinna, precrural area, ventral midline, udder), mucosa, hair, nails, fur, feathers, and comb; *C* mouth; *D* perianal area, anus; *E* eye, conjunctival sac

[a]Key for lipid-dependent yeasts: *1* not identified or not specified; *2 M. sympodialis*; *3 M. globosa*; *4 M. furfur*; *5 M. slooffiae*; *6 M. obtusa*; *7 M. restricta*; *8 M. nana*; *9 M. "equi"* and *M. equina*; *10 M. caprae*

Fig. 3.8 An exfoliative dermatitis caused by non lipid-dependant *Malassezia* yeasts was firstly reported in 1925 by Weidman on an Indian rhinoceros from Philadelphia [204]. In 1996, similar lesions were observed in a Southern white rhinoceros [9]

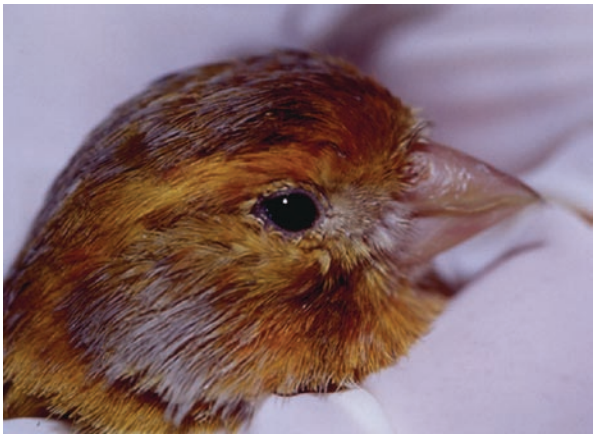

Fig. 3.9 The skin and mucosa of birds may be colonized by lipid-dependent or nonlipid-dependent *Malassezia* yeasts. In the present case, *Malassezia* overgrowth was responsible for the loss of small feathers around the eyes (Service de Parasitologie, ENVA)

was able to grow on ordinary media apparently without any lipid supplementation. Weidman classified this yeast in the genus *Pityrosporum* because of its resemblance to *P. ovale*, the purported agent of human dandruff. However, the regularity in form and the smaller size of the yeast recovered from the Indian rhinoceros led Weidman to describe the species *Pityrosporum pachydermatis*, which was later changed to *Malassezia pachydermatis* [87, 89]. The skin of rhinoceroses seems to be frequently colonized by *Malassezia* yeasts [91, 205].

In 1969, Midgley and Clayton [137] investigated the mycobiota of 90 mammals and 258 birds settled in a zoological park in London. They identified *Malassezia* yeasts in 32.2% of mammals (20% of primates and 56.7% of canines), 30% of water fowls, 2% of tropical birds, and 4.7% of parrots. Dufait [66] isolated *M. pachydermatis* from feathers of 15 birds out of 18 sampled. Another study reported the presence of *M. pachydermatis* in the throat of infected scarlet macaw [29]. Guillot et al. [91] isolated *Malassezia* yeasts from cutaneous samples of six birds (Fig. 3.9). However, a study performed on 103 psittacine birds, both healthy and with manifested feather-destructive behavior, did not recover any *Malassezia* yeasts using cytology examination, nor in fungal cultures of samples taken from the skin surface [169].

Wild animals have always been an interesting source of different microorganisms, including *Malassezia* yeasts [47, 91, 107, 123]. The examination of 165 foxes, one wolf and one bear resulted in the isolation of *M. pachydermatis* from the ears of 13, and anuses of 7 foxes, while one fox harbored the species on both locations. In addition, the same yeast was isolated from

the external ear canal of wolf, and the ear and anus of a brown bear [107]. In 1994, Guillot et al. [91] investigated the prevalence of *Malassezia* yeasts on different animal species, including 356 domestic and wild animals that represented 40 different species. Most of the isolated yeasts belonged to *M. pachydermatis* and originated from carnivores. Lipid-dependent yeasts, which could not be precisely identified at that time, were isolated from domestic animals (viz. cow, sheep, and pigs) and from wild animals (viz. elephant, chimpanzee, rhinoceros, cheetah, and okapi). This study covered also Mammalian orders (Rodents, Lagomorphs, and Insectivores) that yielded negative results. An epidemiological study on 132 wild felids resulted in a the recovery of *M. pachydermatis* in 43.1% of small felids, while *M. sympodialis* was recovered only in large felids with 56.9% positive animals [47]. In Brazil, ear swabs from 30 bats allowed the isolation of *M. pachydermatis* in 62.5%, *M. furfur* in 20.8%, *M. globosa* in 12.5%, and *M. sympodialis* in 4.2% of sampled animals [77]. Despite these studies, the presence of *Malassezia* yeasts on the skin of many wild animals remains largely unexplored.

3.3.2.2 Domestic Carnivores

Malassezia yeasts are frequently isolated from carnivores, especially dogs. In 1955, Gustafson was the first one to isolate lipophilic yeasts from otitis externa (OE) in dogs [104]. He related the isolates to the genus *Pityrosporum* on the basis of their cellular shape and the type of budding, but distinguished them from already described species. In contrast to the other lipid-dependent species, Gustafson's isolates did not require fatty acid supplementation for growth. Gustafson created the new species *Pityrosporum canis* that was made a synonym under *M. pachydermatis*, a combination already proposed by Dodge in 1935 [60] and Gordon in 1979 [83]. Gustafson was able to induce OE by applying *M. pachydermatis* to the external ear canal, whereas lipophilic yeasts isolated from humans had no effect. Fraser [69] isolated *M. pachydermatis* in comparable frequencies from healthy dogs and those with OE, but found that affected dogs tended to have larger numbers of the yeast on smears of cerumen. Over the last 10 years, in addition to *M. pachydermatis*, some lipid-dependent species were recovered from dog skin, but, unfortunately, the identification method used was not sufficiently clear [85, 172, 173] or lipid-dependent yeasts were not identified [34]. Cafarchia et al. [34] isolated lipid-dependent species from healthy dogs and Crespo et al. [50] sampled atypical lipid-dependent species from dogs that suffered from chronic OE. However, in these last studies no molecular identification was performed. Two years later, Duarte et al. [63] isolated atypical lipid-dependent yeasts from the ear of a healthy dog.

Among the different *Malassezia* species, the non lipid-dependent *M. pachydermatis* can be isolated from the external ear canal and mucosa of healthy cats and those with OE and dermatitis [91]. Generally, *M. pachydermatis* is more frequently isolated from dogs than from cats. In healthy cats, the reported percentages of isolation of this species range from less than 10% [25, 109] to approximately 20% [52, 59, 91, 147] and up to 40% [33]. However, *M. pachydermatis* has been identified in 41–71% of cats with OE [33, 52, 59, 147].

Although lipid-dependent species have also been reported from canine specimens, they seem to occur more frequently in cats than in dogs. The presence of lipid-dependent *Malassezia* yeasts in healthy carnivores was initially demonstrated in felines. *M. globosa* was isolated from normal skin of a cheetah (*Acinonix jubatus*) [87, 91]. Lipid-

Fig. 3.10 The skin of Devon Rex cats represents a favorable biotope for the development of *Malassezia* yeasts. In this breed, high numbers of *Malassezia* yeasts were detected in different anatomic sites, including axilla, groin, ventral neck, and nail folds (Service de Zootechnie, ENVA)

dependent species, such as *M. furfur* [48, 147], *M. globosa* [25, 33, 147], and *M. sympodialis* [23, 47, 52, 59], have also been reported from the skin or the external auditory canal of healthy pet cats or other felids. On the other hand, Colombo et al. [45] recently found that *M. pachydermatis*, *M. furfur*, and *M. sympodialis* can be frequently isolated from the nail folds of healthy cats of various breeds, especially in Devon Rex cats (Fig. 3.10). These authors pointed out that the presence of a high number of yeasts in cytological investigations correlated with the clinical observation of the presence of brown, greasy material in the nail folds. *Malassezia* overgrowth was also reported in allergic cats, mainly with AD [155].

Most of these isolates, however, have been identified on the basis of phenotypic characteristics and misidentification might occur. The speciation of lipid-dependent isolates from animals by means of physiological tests presents some difficulties. Crespo et al. [49] reported for the first time lipid-dependent yeasts associated with OE in cats. These yeasts had similar morphological characteristics and some shared physiological characteristics with the type strain of *M. sympodialis*. More recently, Hirai et al. [113] described *M. nana*, a novel species from otic discharges of a cat and cows. The type strain of *M. nana* showed identical physiological characteristics to those of *M. sympodialis*, but with the only exception being the presence of ring-like growth with Tween 20 [112]. In fact, the frequently reported isolates from feline skin that showed a similar phenotype to *M. sympodialis* may be conspecific with *M. nana*. Some lipid-dependent strains isolated from cats that were found to be similar to the type strain of *M. sympodialis* were studied using DNA sequence analysis and it was found that they grouped together with the *M. nana* AB075224 LSU

rRNA gene sequence [30]. *M. nana* seems to be the most common lipid-dependent species isolated from cats. This species and *M. slooffiae* have recently been isolated from cats with different disorders [159], and *M. slooffiae* has been found in the claw folds of healthy and seborrhoeic Devon Rex cats [1].

3.3.2.3 Domestic Herbivores

Lipid-dependent species constitute the major population of the lipophilic mycobiota in herbivores, such as horses, goats, sheep, and cows [51] and their occurrence in these animals was much greater than that of *M. pachydermatis*. Different species, such as *M. furfur, M. globosa, M. obtusa, M. slooffiae, M. sympodialis*, and *M. restricta*, were phenotypically identified, but they were not confirmed by rDNA sequence analysis. Moreover, a large number of isolates could not be identified by means of phenotypic techniques.

A lipid-dependent isolate identified as *M. furfur sensu lato* was recovered from the healthy skin of a cow [91]. A strain from the normal ear of a cow and a strain of the normal skin of a cow were included in the description of *M. furfur sensu stricto* and *M. globosa,* respectively [87]. Different lipid-dependent species have also been recovered from healthy cattle from Minas Gerais, Brazil [61, 63, 64], namely *M. furfur, M. globosa, M. obtusa, M. sympodialis, M. slooffiae*, and *M. globosa*, which were phenotypically identified from cerumen of the external ear of these animals. The most common species isolated from these animals was *M. sympodialis*. However, the identity of these isolates was not confirmed by rDNA sequence analysis. Four lipid-dependent strains from cows were considered to be genetically identical to the type strain of *M. nana* [113]. Molecular analysis showed, however, that some differences occurred between the strain from cat and those from cow, respectively.

Nell et al. [149] reported the presence of a novel *Malassezia* species from normal equine skin, which they tentatively named "*Malassezia equi*," but without including a valid description. The species was identified as a member of the genus *Malassezia* by sequence analysis of the D1/D2 domains of the LSU rRNA gene, and was found to be closely related to *M. sympodialis*. In a study of the lipophilic mycobiota of the intermammary and preputial fossa areas of horses "*Malassezia equi*" and *M. slooffiae* were identified by PCR sequencing analysis [207]. In another study, several isolates from horses that phylogenetically were related to *M. sympodialis* grouped together by D1/D2 and ITS sequence analysis and were described as *M. equina* [30, 31].

Few reports are available on *Malassezia* yeasts that affect the skin of goats [12, 200]. Lipid-dependent isolates identified as *M. furfur sensu lato* were recovered from the healthy skin of two sheep as well [91]. In the last revision of the genus, two strains from the normal skin of a goat and a sheep were included in the description of *M. slooffiae* [87]. Lipid-dependent *Malassezia* species, such as *M. furfur*, *M. globosa*, *M. obtusa*, *M. sympodialis*, and *M. restricta*, have also been isolated from the skin of healthy goats, and *M. globosa, M. restricta*, and *M. sympodialis* were obtained from the skin of healthy sheep. These isolates were phenotypically identified, but they were not confirmed by rDNA sequence analysis [51]. From these isolates, the sequence of the D1/D2 domains of the 26 rRNA of a lipid-dependent strain from sheep was found to be

identical to that of the type culture of *M. sympodialis* [30]. *Malassezia caprae*, a lipid-dependent species isolated mainly from healthy goats, has been recently described as well [31].

3.3.2.4 Swine

In 1959, Gustafson isolated *Malassezia* yeasts from three out of 32 swine [105]. In 1985, Dufait [66] successfully cultured *M. pachydermatis* from six pigs. The isolation of *Malassezia* lipid-dependent yeasts from 23 out of 40 [91] and 73 out of 100 swine [78] suggested that this animal species is regularly colonized by lipophilic yeasts. Garau et al. [78] reported colonization of swine ears with *M. sympodialis* and *M. slooffiae*, while swab and carpet did not reveal any *Malassezia* yeasts from anus, forehead, and back [78]. Another study [163] reported the presence of nonlipid-dependent strains in swine ears. Some of these isolates were randomly chosen and their identification as *M. pachydermatis* was confirmed by karyotyping and restriction fragment length polymorphism analysis.

3.3.2.5 Rodents and Lagomorphs

Dufait isolated nonlipid-dependent *Malassezia* yeasts from guinea pigs and rabbits [66]. However, further investigations failed to demonstrate the presence of lipophilic yeasts in rodents or lagomorphs [75, 91]. Recently, Galuppi et al. [75] examined 147 laboratory rats and did not isolate any yeast.

3.3.2.6 Distribution Within a Single Animal Host

Because of the importance of *Malassezia* dermatitis in dogs, many investigations have been conducted in order to describe the distribution of *M. pachydermatis* on the body of dogs. Lipophilic yeasts can be isolated from skin, auditory canal, anus, oral cavity, lips, and mucosae of male and female genitalia (vagina, preputium) [181]. Colonization of the canine skin probably occurs in the very first days after birth, but the way by which colonization occurs is still not understood. Wagner and Schadler [203] isolated *Malassezia* yeasts in 39.4% of all samples collected from the lips, nail beds, and ears of 3-day old Rottweilers. The percentage increased to 41.2% in 1-week old puppies, and decreased to 37% when the animals were 5 weeks old. Most of the samples recovered from dams yielded negative results, thus excluding the possibility that *Malassezia* was transferred through cleaning and feeding processes [203]. Oral carriage was investigated by Bond and Lloyd [19] who compared two different antifungal shampoos against SD in basset hounds. Application of the miconazole-chlorhexidine shampoo throughout a 3-week

period resulted in a significant reduction of the *Malassezia* population on both the skin and in the mouth. However, the importance and significance of oral transfer remained unclear. Another study pointed out the possible transfer of *Malassezia* yeasts from pruritic skin lesions of the inguinal area to the perioral area as a consequence of frequent licking. In addition, dogs with lesions in the perioral area yielded a greater number of *Malassezia* yeasts in the interdigital region without visible skin damage as a probable result of persistent scratching [34].

Different isolation techniques were used to assess the number of *Malassezia* and to determine a possible threshold value that could trigger *Malassezia* dermatitis. It is well known that sampling techniques determine the detection limit of *Malassezia* yeasts. Kennis et al. [119] reported that at least one positive *Malassezia* sample could be detected in 95% of examined dogs when using the culture method, while cytological examination of samples collected by the direct impression method using the glass slide, cotton swabs, and skin scraping techniques yielded positive results in 74% of dogs. Evaluation of these three techniques separately yielded positive results in only 37–57% of the dogs [119].

The results of epidemiological surveys also rely on the choice of the culture conditions. *M. pachydermatis* is considered a nonlipid-dependent species, which grows well on Sabouraud glucose agar. However, the use of specific media, such as modified Dixon medium, showed a significant higher recovery rate of lipophilic yeast in comparison with that on Sabouraud glucose agar [95]. Until 1995, it was believed that dogs were carriers of *M. pachydermatis*, a species that does not require lipids in media for their growth. Transfer of some *M. pachydermatis* colonies from modified Dixon agar to Sabouraud's glucose agar, however, showed fastidious growth and the need for the addition of lipids. Karyotyping of those isolates confirmed a typical *M. pachydermatis* pattern [16], and two years later partial sequencing of the LSU rRNA defined them as sequence type Id [92]. In 1999, Senczek et al. [182] distinguished two different types of colonies of *M. pachydermatis* isolated from inflamed external ear canals of dogs. Small colonies showing poor expansion growth on culture media reached only 1 mm in diameter after 72 h of incubation, whereas bigger ones showed faster growth and colonies of 4 mm in diameter. Ahman et al. [1] isolated two different types of *M. pachydermatis* in Devon Rex cats. One type showed good growth on Sabouraud glucose agar, while colonies of the other type remained significantly smaller, and were harder to grow on Sabouraud glucose agar.

Several investigations explored *Malassezia* colonization of various anatomical regions in different breeds of healthy dogs. Results differed from 10 to 100% and depended on the dog breed, sampled anatomic location, and identification method used [93]. Most frequently sampled sites were the external ear canal, anus, followed by mouth, buccal mucosa, haired skin of lips, pinna, dorsum, axilla, groin, interdigital area, nail beds, prepuce, and vulva [22, 33, 34, 81]. *M. pachydermatis* has been identified in 57.8% of clinically healthy dogs [108]. Lukman [127] reported that 56.6% of anal sacs were colonized with *M. pachydermatis*, followed by the auditory canal (48.6%), rectum (46%), anus (40.6%), and vagina (38.2%). A study performed on 20 beagles and 20 pet dogs of various breeds and crossbreeds showed significant differences in the number and occurrence of *Malassezia* isolation between beagles and the other breeds of dogs [22]. In 55% of examined beagles *Malassezia* prevailed in the anus and ears, while in pet dogs it prevailed in anus (50%) and mouth (30%). In pet

dogs, the nose, prepuce, and vulva were colonized with lower frequency and were positive in 8.5–10% of the animals investigated. A similar situation was observed in beagles where frequencies ranged from 0 to 20%. Beagles had statistically a significant higher number of *Malassezia* yeasts than pet dogs (55 vs. 10%) in terms of population size and frequency of isolation from the ear. It is notable that isolation attempts from dorsum samples of both pet dogs and beagles remained negative. Moreover, the lower lip region was colonized with *Malassezia* in the range of 75–80%, and the interdigital area from 60 to 70% of the samples from healthy dogs [22]. A similar study was carried out on healthy basset hounds and resulted in the recovery of *M. pachydermatis* from the mouth (92.3%) and vulva (80%). The axilla was colonized in all dogs, the nose in 69.2%, and this was significantly higher than the values obtained from healthy mixed-breed dogs. By comparison, *Malassezia* yeasts were recovered from the axilla in 15%, from the mouth in 30%, the nose in 10%, and from vulva in 8.5% of mixed-breeds [18]. Comparable results were reported when colonization of *M. pachydermatis* was investigated of the hair of the lower lip and the hair follicles of healthy beagles. *M. pachydermatis* was most frequently isolated in hair samples from the lips (75% of beagles), whereas isolations from the fore foot and dorsum were found to be positive in 33% of animals [95]. An investigation conducted in France reported that 91% of the samples recovered from dogs' lips and 43% from their ears were positive in culture for *M. pachydermatis* after cytological examinations yielding 3.1 and 2.2 *Malassezia* cells per field [95]. In the study by Bensignor et al. [11], pinna was the area most frequently colonized with *Malassezia* yeasts. Contrary to previous studies, lower detection and isolation frequency were observed in the perianal area when compared with the umbilical and axilla areas [11]. Kennis et al. [119] reported the highest number of *Malassezia* yeasts in the chin region, while the inguinal and axillary zones presented the lowest number. A study on 99 healthy dogs revealed the prevalence of *M. pachydermatis* in the ear of 39.4% of cytologically examined samples and 45.5% of samples that were identified by culture [121]. Cafarchia et al. [34] isolated *Malassezia* yeasts in the perianal area of 60.6% from healthy dogs, followed by 36.4% of the perioral area from healthy dogs. The interdigital area, ear canal, and periorbital sites were found to be less frequently colonized with 18.2, 12.1, and 9.1% of the animals investigated, respectively. The inguinal area was positive in only 3% of dogs [34]. Girão et al. [81] found *M. pachydermatis* in 30% of the ears of healthy dogs after microscopic and culturing assessment. Cytological quantification of *Malassezia* in the anal sac content of healthy dogs revealed a low occurrence of yeasts, with only 12.5% of the examined dogs and 10% of the examined anal sacs demonstrating the presence of *Malassezia* yeasts [156]. Although there is no consistent pattern in the results obtained from several cases for which the anal and perianal regions were examined, the perioral area, including lips and mouths, ear skin and ear canal, were most frequently colonized with *Malassezia* yeasts. Finally, all studies resulted in the same conclusion, namely that *Malassezia* colonization occurs more frequently in the hairy areas that are rich with sebaceous glands [93].

Disturbance of the mutual coexistence between the animal and *Malassezia* mycobiota can lead to *Malassezia* overgrowth and development of skin disorders, such as dermatitis, paronychia, facial or pedal pruritis, and OE in dogs [142]. In order to detect an infection caused by *Malassezia*, it is important to gain insight into the population sizes of *Malassezia* on healthy dog skin and mucosa and their ecology [93], because there is no uniform threshold value of the number of *Malassezia* yeasts that will result in exacerbation of skin disease.

The presence of viable *Malassezia* yeasts in the environment is considered to be low. The environment has, at least, an anecdotic role in our understanding of the epidemiology of *Malassezia*-related diseases. *Malassezia* yeasts have been detected in sand [131] and water (G. Midgley, personal communication). Renker et al. [175] detected DNA of lipid-dependent *Malassezia* yeasts (viz. *M. globosa* and *M. restricta*) from nematodes living in soils from forests in Central Europe. The biological significance of the possible presence of *Malassezia* yeasts in the soil remains to be investigated.

3.3.3 Factors Associated with Increased *Malassezia* Populations in Animals

3.3.3.1 Factors Related to the Animal Hosts

Many factors may be associated with the proliferation of *Malassezia* yeasts in animals, including breed, gender, and age. Moreover, alteration of microenvironmental skin conditions connected to a chemical mechanism (pH modifications), long-term treatments (antibiotics and glucocorticoids), or presence of other bacteria or fungi and coexisting diseases may contribute to *Malassezia* proliferation. Numerous studies recognized breed predisposition in dogs as an important factor. In 1982, Lukman [127] noted the highest frequency of *Malassezia* recovery in Dalmatian dog, German shepherd, and German shorthaired pointer. Hajsig et al. [108] reported that *M. pachydermatis* was found in 75% of examined German shepherd dog breed. Cytological examination of 39 different breeds of dogs resulted in a significantly higher prevalence of *Malassezia* in basset hounds and dachshunds when compared with all other breeds of dogs examined in the study. A similar, but statistically not significant tendency was observed in cocker spaniels, springer spaniels, and German shepherds [165]. Bond et al. [24] reported that basset hounds, cocker spaniels, and West Highland white terriers are breeds predisposed to develop *Malassezia* dermatitis (Fig. 3.11). Another study on healthy basset hounds showed a significantly higher number of *M. pachydermatis* recovered from the nose, mouth, and vulva in comparison with healthy mixed-breed dogs [18] (Fig. 3.12). In the

Fig. 3.11 West Highland white terriers are predisposed to *Malassezia* dermatitis (Service de Parasitologie, ENVA)

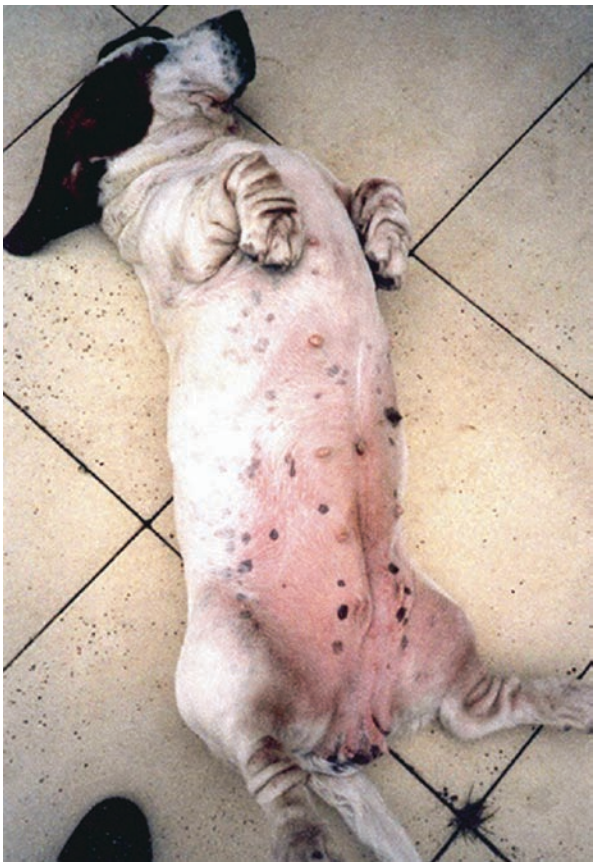

Fig. 3.12 The skin of basset hounds represents a very favorable biotope for the development of *Malassezia* yeasts (Service de Parasitologie, ENVA)

study exploring immunological responses of healthy dogs and dogs with dermatitis, a significantly higher number of *M. pachydermatis* was found in axilla of healthy basset hounds in comparison with beagles and setters [26]. Kumar et al. [121] reported the highest presence of *Malassezia* otitis in German shepherds and Labrador retrievers. Girão et al. [81] showed the highest prevalence of otitis connected to *M. pachydermatis* in the poodle. A large number of healthy German shepherds were found positive for *M. pachydermatis*. An epidemiological study on 1,370 dogs reported a higher occurrence of OE in breeds with pendulous ears when compared with breeds with erect ears [133]. Nobre et al. [152] reported a higher occurrence of *Malassezia* otitis in cocker spaniels, German shepherds, and Brazilian fila, while Mauldin et al. [136] found that West Highland white terriers, English setters, Shih Tzus, basset hounds, and American cocker spaniels were predisposed to *Malassezia* dermatitis. Some breed predispositions are related to physical characteristics of the skin, such as the presence of skin folds in basset hounds [18, 97] or pendulous ears [33, 81, 133, 168].

Several studies, performed to determine a possible age predisposition in dogs, reported contradictory results. A few investigations did not find a significant correlation between the number of *Malassezia* and the age of the animal [152, 165]. Other studies demonstrated that the age of the dogs could be considered as a predisposing factor, but the conclusions of these

investigations were not the same. In a study by Mauldin et al., [135] more than 70% of dogs with *Malassezia* dermatitis were 4 years and older. Girão et al. [81] reported the highest occurrence of *Malassezia* otitis in 1–3-year-old dogs, Kumar et al. [121] in 2–4-year-old dogs, and Cafarchia et al. [33] detected higher *Malassezia* populations in dogs younger than 1 year.

Gender predisposition has been excluded by numerous studies [81, 121, 152, 165]. In most cases, equal numbers of *Malassezia* yeasts occurred in females and males (46.43 vs. 53.57%) as reported by Girão et al. [81]. However, studies conducted in Italy [33] observed a higher number of *Malassezia* recovered from males than from females with otitis, and a study from Brazil reported 60.7% of positive males in comparison with 33% of the females [169], thus indicating possible gender predisposition. In a retrospective study of *Malassezia* dermatitis in dogs, it has been reported that sterilized females and males were more susceptible to develop this skin disorder [135].

3.3.3.2 Factors Related to the Skin Microenvironment

The optimal temperature for *Malassezia* growth under laboratory conditions is 32–34°C. Some species can grow below these values (viz., *M. pachydermatis*); others develop at 37°C or above (viz. *M. pachydermatis*, *M. sympodialis*, *M. furfur*, *M. yamatoensis*, *M. slooffiae*, and *M. dermatis*). For optimal growth of *Malassezia* yeasts, an adequate pH value is required. It was reported that optimal pH values for successful *Malassezia* development range between 4.0 and 8.0 [134]. The highest occurrence of canine *Malassezia* otitis in India was reported in June with 15.2% of otitis cases [121]. On the contrary, Cafarchia et al. [33] reported higher recovery of *Malassezia* yeasts from dogs with otitis during the winter. In general, there are indications that warm and humid weather supports the exacerbation of *Malassezia* infection in animals.

Malassezia pachydermatis demonstrates a preference toward the external ear canal causing OE in many cases. Certain conditions in the host need to be satisfied to stimulate the growth of this opportunistic pathogen. The ear canal is a dark, humid place, rich with cerumen, and experiencing moderate air circulation depending on the ear shape. Such an environment represents an optimal place for the exacerbation of infection. The concentration of fatty acids in the earwax influences the susceptibility to OE [133]. Most dog breeds with pendulous ears showed significant higher amounts of fatty acids in the ear (with the exception of the pug) than breeds with erect ears, when Siberian husky was excluded from this group [133].

Besides the mentioned physical and chemical factors that enable the attachment of *Malassezia* and the consequent infection, other conditions that may contribute to *Malassezia* overgrowth are: hormonal disbalance, keratinization defects, excessive production of sebum, bacterial infections, and hypersensitivity processes [181].

Atopic dermatitis is a common skin disease from which *Malassezia* yeasts can be recovered in high numbers. However, a significant association between atopic lesions and *Malassezia* colonization has not been found, suggesting that yeast overgrowth occurs secondarily as a result of changes in the skin microenvironment [148]. Regarding the location, the highest recovery of *Malassezia* yeasts was observed in the interdigital areas of the skin with 70.7% of isolations, followed by the ears with 63.4%. Nail folds, mouth, and groin were places with lower frequency of *Malassezia* isolation, approximately 33% positive isolations on average [148].

Different immunodeficiency status may be connected to malfunction of the immune system and inadequate immunologic responses to *Malassezia* yeasts. Chen and Hill [43] described immunological responses that occurred after the interaction of *M. pachydermatis* antigen and the skin immune system in dogs. Induction of Th1 lymphocytes or cell-mediated response leads to the production of IL-2 and IFN-γ cytokines, thus promoting the release of immunoglobulin IgG, while the induction of Th2 or humoral response leads to production of IL-4 and IL-13, followed by immunoglobulin IgE [43]. In the last decade, different surveys have been performed in order to explore cellular and humoral immune responses to *M. pachydermatis* in dogs. In an *in vitro* study, the possibility of proliferation of peripheral blood mononuclear cells as a result to *M. pachydermatis* antigen was explored. The results showed that dogs with and without incidence of disease were able to develop cell-mediated response to *M. pachydermatis* antigen. Additionally, this study indicated that there was no connection between low levels of total IgA in sera of dogs and the presence of high population densities of *M. pachydermatis*. Finally, high levels of specific IgG were found in the serum of dogs affected with *Malassezia* dermatitis [26].

3.3.3.3 Factors Related to *Malassezia* Species and Isolates

The evaluation of enzymatic activities of 33 different *Malassezia* strains belonging to various species showed that strains of *M. pachydermatis* had the highest enzymatic activity as they were capable to secrete 15 different enzymes. *M. furfur* produced eight, *M. sympodialis* and *M. slooffiae* five, *M. obtusa* four, and two *M. globosa* strains three and four enzymes, respectively. *M. restricta* secreted only naphtol-AS-BI-phosphohydrolase, thus demonstrating a narrow range of enzyme production [130]. Isolates of *M. pachydermatis* obtained from dogs with single or generalized skin lesions demonstrated significantly higher phospholipase activities than those obtained from healthy skin [38], thus suggesting the importance of enzymatic activity in the development of cutaneous *Malassezia* overgrowth.

In epidemiological studies, genotyping of *Malassezia* yeasts may be required in order to identify the source of infection and to discover a possible connection between genotypes and disease. Different studies targeted different rRNA or rDNA regions in order to distinguish different molecular patterns among *M. pachydermatis* resulting in a genotypic classification. Sequence analysis of the D1/D2 domains of the LSU rRNA of 100 isolates of *M. pachydermatis* resulted in the differentiation of seven different genotypes (or sequence types), namely, Ia, Ib, Ic, Id, Ie, If, and Ig [92]. Sequence types Ic, Id, and I g seemed to be specific for rhinoceros, dogs, and ferrets, respectively. Sequence type Ia was found in isolates from both animals and humans, while sequence type Id involved *M. pachydermatis* showing small colonies and poor growth on Sabouraud glucose agar. In general, all *M. pachydermatis* isolated from dogs belonged to sequence types Ia, Id, and Ie. Moreover, it was found that one animal could be the carrier of two or more *Malassezia* sequence types [92]. Sequence analysis of *M. pachydermatis* isolates from the external ear and cutaneous lesions of sea lion using the variable domains of the LSU rRNA showed that all strains belonged to sequence type Id [94].

Aizawa et al. [2] used random amplification of polymorphic DNA (RAPD) and chitin-synthase gene (*CHS2*) sequencing in order to discriminate 16 isolates of *M. pachydermatis*. Both methods yielded similar results, namely A, B, and C sequence types, and 13 isolates matched with the type strain of *M. pachydermatis*. Subsequently, the same research group used the same molecular method to differentiate 98 *M. pachydermatis* isolates from dogs and 12 from cats. RAPD analysis yielded four different genotypic groups of *M. pachydermatis* listed A, B, C, and D. RAPD type A was identified as the most common genotype and occurred in 81 isolates from dog skin and ear and was found to be identical to *M. pachydermatis*, while RAPD type B pattern was observed in 13 isolates and was close to *M. furfur*. RAPD type C of *M. pachydermatis* showed a different sequence pattern and was isolated only from one dog suffering from OE. RAPD type D was found in two isolates of dogs with OE and one with AD. The 12 isolates from ear and skin form cats belonged to RAPD type A [3].

Sugita et al. [192] explored the variable intergenic spacer region (IGS1) as a possible genotypic marker for *M. pachydermatis*. Forty-three strains isolated from carnivores (dogs and cats) were analyzed and three different groups with ten subtypes were identified. The authors stated that their IGS groups 1, 2, and 3 matched with the sequence types Ie, Id, and Ia as identified previously by Guillot et al. [92, 192].

RAPD analysis was performed by Castellá et al. [40] in order to genotype isolates of *M. pachydermatis* originating from dogs, cats, horses, goat, and pig. Thirty five from 55 isolates belonged to RAPD type I showing 12 bands. RAPD types II, III, and IV were found only among dog isolates. RAPD types II and IV displayed nine bands and were recovered from two animals that suffered from otitis, and RAPD type III had 11 bands and was isolated from a healthy dog. The authors suggested that their RAPD type I corresponded to sequence type Ia as determined by Guillot et al. [92] or RAPD type A as described by Aizawa et al. [3]. In addition, they reported that one animal can host different genotypes of *M. pachydermatis* [40].

Cafarchia et al. [36] analyzed 104 *Malassezia* isolated from dogs using sequence analysis of three different loci, namely *CHS2*, the LSU rRNA gene, and the ITS1 region of the rDNA. The authors distinguished three different sequence types: A, B, and C, but analysis of the ITS-1 region yielded an additional sequence type. In 185 samples of *M. pachydermatis* isolated from 30 healthy and affected dogs, three *CHS2* (A_c, B_c and C_c) and eight ITS1 (A_{I1}–A_{I4}, B_{I1}, C_{I1}–C_{I3}) genotypes and subgenotypes were identified [38]. All these observations indicate the presence of a considerable genetic diversity among *M. pachydermatis*, which, at least in part, may be relevant for our understanding of the interplay between the yeast and the patient.

3.4 Conclusions

In the last decade, a broad variety of *Malassezia* identification and (bio)typing tools has emerged. Owing to the different growth requirements by *Malassezia* spp. culture-independent molecular methods seem most accurate to assess the diversity of *Malassezia* yeasts in culture, on human, or animal skin. A wide array of PCR-based methods, such as

RAPD, PCR-typing, *t*FLP, RFLP, and PCR-derived methods comprising DGGE, SSCH, and the application of Luminex technology, has emerged to answer different research questions. In addition, the usefulness of the analysis of clone libraries and real-time PCR with clinical specimens is demonstrated in numerous studies. Recent advances in *Malassezia* genomics should pave the way for the design of a Multi Locus Sequence Typing (MLST) scheme for the various *Malassezia* species. On another track, further automation in the molecular analysis of *Malassezia* mycobiota can be expected due to the development of diagnostic (micro-)array platforms.

Detection and identification of *Malassezia* species from clinical specimens by real-time PCR has indicated that the species are present in related dermatoses. *M. globosa* and *M. restricta* are the most common species in pityriasis versicolor (PV), seborrheic dermatitis (SD), atopic dermatitis (AD), and psoriasis (PS). However, the ratio of these two microorganisms differs depending on the disease psoriasis. Recent qualitative and quantitative studies suggest that *M. restricta* plays a significant role in SD and PS, *M. globosa* plays a significant role in PV, while both species may be responsible for exacerbating AD.

Malassezia yeasts may be isolated from the skin and mucosa of a very wide range of warm-blooded vertebrates (all the mammals and probably many birds). However, the prevalence of colonization and the density of *Malassezia* populations vary according to the animal species and the anatomical sites. The nonlipid-dependent species *M. pachydermatis* is a common inhabitant of skin and mucosa in dogs. The mean carriage of lipophilic yeasts varies from 10 to 100% and depends on the dog breed, the anatomical location, and the identification methods used in the studies. The presence of lipophilic yeasts is more rarely reported in other animals and the *Malassezia* species involved may be different. Although *M. pachydermatis* is found in the external auditory of cats, this does not occur with the same high frequency as in dogs. Lipid-dependent species seem to be more frequent in cats than in dogs. Lipid-dependent species are the major component of the cutaneous microbiota in ruminants and horses. In swine, both lipid-dependent and nonlipid-dependent species have been isolated. Such a complex situation is probably related to the specific composition of cutaneous lipids and the competition with different types of microbiota (e.g., bacteria and yeasts) within the skin ecosystem. The recent analysis of the genome and secretory proteome of *M. globosa* and *M. restricta* showed that their lipid dependence can be attributed to the absence of a fatty acid synthase gene, while harvest of host lipids can be aided by several secreted lipases. Extending genomic and proteomic research to all known *Malassezia* species, and comparing the lipid composition on the epidermis of the corresponding hosts should enhance our understanding of *Malassezia* biology and explain the role of the various species in the disease of different animals.

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