Biodiversity, Phylogeny and Ultrastructure

2

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Core Messages

- This chapter presents and discusses all techniques and media used to isolate, maintain, preserve, and identify the 13 species that are presently included in the genus. Each species is described morphologically, including features of the colonies and microscopic characteristics of the yeast cells, either with or without filaments; physiologically, including the growth at 37 and 40°C, three enzymatic activities, namely catalase, β-glucosidase and urease, and growth with 5 individual lipid supplements, namely Tween 20, 40, 60 and 80, and Cremophor EL. Their ecological preferences and role in human and veterinary pathology are also discussed.
- For quite a long time, the genus was known to be related to the Basidiomycota, despite the absence of a sexual state. The phylogeny, based on sequencing of the D1/D2 variable domains of the ribosomal DNA and the ITS regions, as presented in the chapter, confirmed the basidiomycetous nature of these yeasts, which occupy an isolated position among the Ustilaginomycetes. The relationship to the Basidiomycetes is also supported by monopolar and percurrent budding and the multilamellar cell wall ultrastructure. Some characteristics of this cell wall, which is unparalleled in the world of fungi, together with the lipophily demonstrate the uniqueness of this genus in the fungal kingdom.

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2.1 Isolation, Identification and Biodiversity of *Malassezia* Yeasts

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The genus *Malassezia* was created for a fungus, *M. furfur*, which was seen in lesions of pityriasis versicolor (PV). Unfortunately, it took a long time to understand the lipid dependence of this fungus and, consequently, to obtain and maintain its culture *in vitro*. Due to the lipid requirements, conventional laboratory techniques used for the identification of yeasts could not be applied to this fungus. Despite the description of numerous species, their accurate identification was not feasible, and the taxonomy of the genus remained a controversial subject for decades. The development of molecular techniques allowed the unequivocal separation of species, and then new laboratory methods were developed to characterize these taxa.

2.1.1 Isolation of *Malassezia* Yeasts from Humans and Animals and their Maintenance

The genus *Malassezia,* created by Baillon in 1889 [1] and also known under the generic name *Pityrosporum* created by Sabouraud in 1904 [2], comprises lipophilic and lipid dependent yeasts that require long chain fatty acid (C12 up to C24) supplementation to grow and survive. Slooff [3] in her overview of the history of the genus considered that Panja had been the first to obtain a culture of *Malassezia* on Petroff's egg medium with 0.004–0.005% gentian violet [4, 5]. Shifrine and Marr [6] obtained cultures by adding several fatty acids, in particular oleic acid, to Sabouraud agar. These media, however, were disappointing, because growth was inconstant and resulted in rapid loss of cultures (see Chap. 1). Van Abbe [7] was more successful when he recommended the complex medium created by Dixon. This Dixon's agar (DA) is still in use, according to its original formula, or in a modified version (modified Dixon agar, mDA) as proposed by Midgley [8]. Next to DA, Leeming and Notman [9] proposed a medium, Leeming and Notham agar (LNA) that allowed growth of these nutrient-demanding microorganisms. This medium, elaborated after testing the different compounds separately, allows for isolation and maintenance of all *Malassezia* yeasts. Therefore, it is now largely used by most researchers working with *Malassezia* yeasts. All these complex media contain Ox bile, but the LNA replaces Tween 40, used in the Dixon formula, by Tween 60. According to the assimilation pattern of the 13 species presently described (Plates 2.1 and 2.2), Tween 60 seems to be more efficiently utilized, thus favoring growth of most species, whereas Ox bile, as demonstrated by Japanese authors [10], is an essential, if not sufficient compound, for good growth of *Malassezia* yeasts. Even *M. pachydermatis,* the less demanding species, requires growth media that are enriched with peptone (i.e., Sabouraud medium), which contains short chain fatty acids. On such media, however, viability is lost rapidly, except if the culture is transferred regularly (about every month). Lorenzini and de Bernadis [11] showed that the addition of Tween 80 enhanced the isolation of *M. pachydermatis* from clinical materials significantly.

Malassezia yeasts belong to the normal cutaneous mycobiota of humans and animals, and the skin lipids most likely contain the nutrients required. The optimal growth

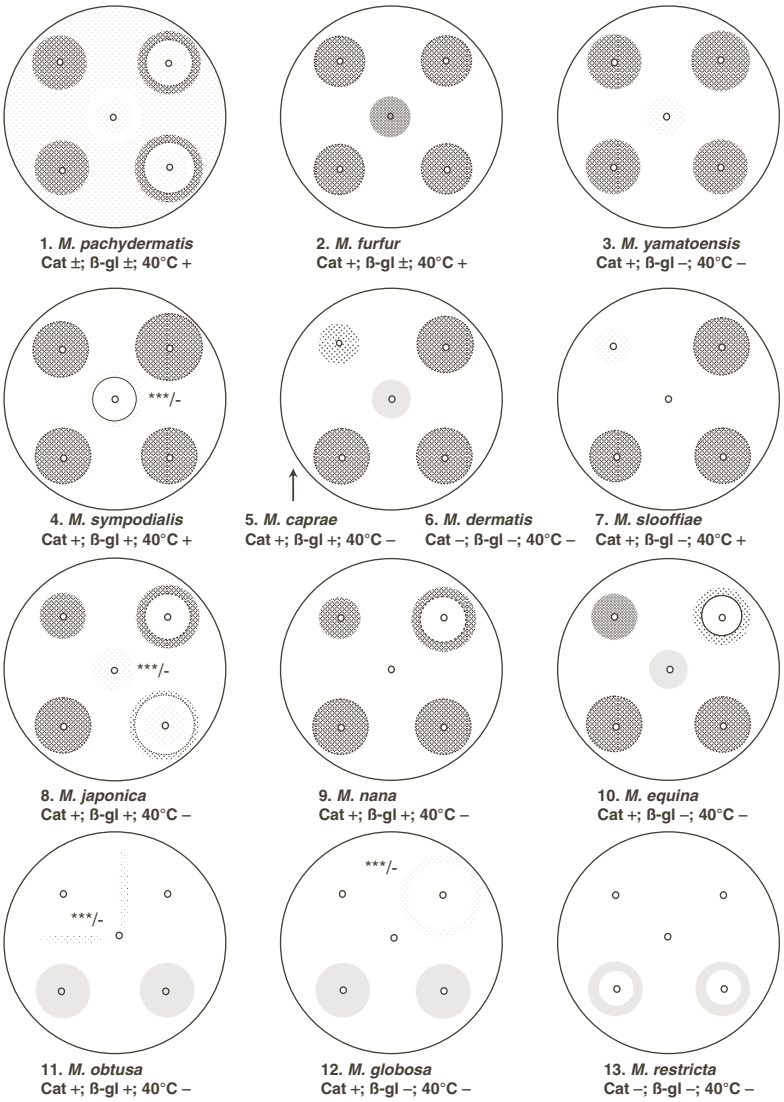


Plate 2.1 Key characteristics of *Malassezia* species, 1 *M. pachydermatis* T; 2. *M. furfur* T; 3. *M. yamatoensis* T; 4. *M. sympodialis* T; 5. *M. caprae* T; (6). *M. dermatis* (not drawn) T; 7. *M. slooffiae* T; 8. *M. japonica* T; 9. *M. nana* T; 10. *M. equina* T; 11. *M. obtusa* T; 12. *M. globosa* T; 13. *M. restricta* T. The 13 species are arranged from the left to the right according to their decreasing physiological and biochemical capacities. **O**: well, 2 mm diameter; top right Tween 20, clockwise Tween 40, 60, 80, Cremophor EL in the centre; : growth very weak or delayed secondary growth within the inhibition area after diffusion of the supplement (*M. pachydermatis* and *M. japonica*); : mild growth and growth of *M. pachydermatis* on GPA, apart of lipid supplements; : very good growth; : precipitate within the agar around the lipid supplements, -/***: absence of growth or colonies present only with recent isolates; *Cat*: catalase activity; *ß-gl*: ß-glucosidase activity; 40°C: growth at 40°C, T: type strain of the species

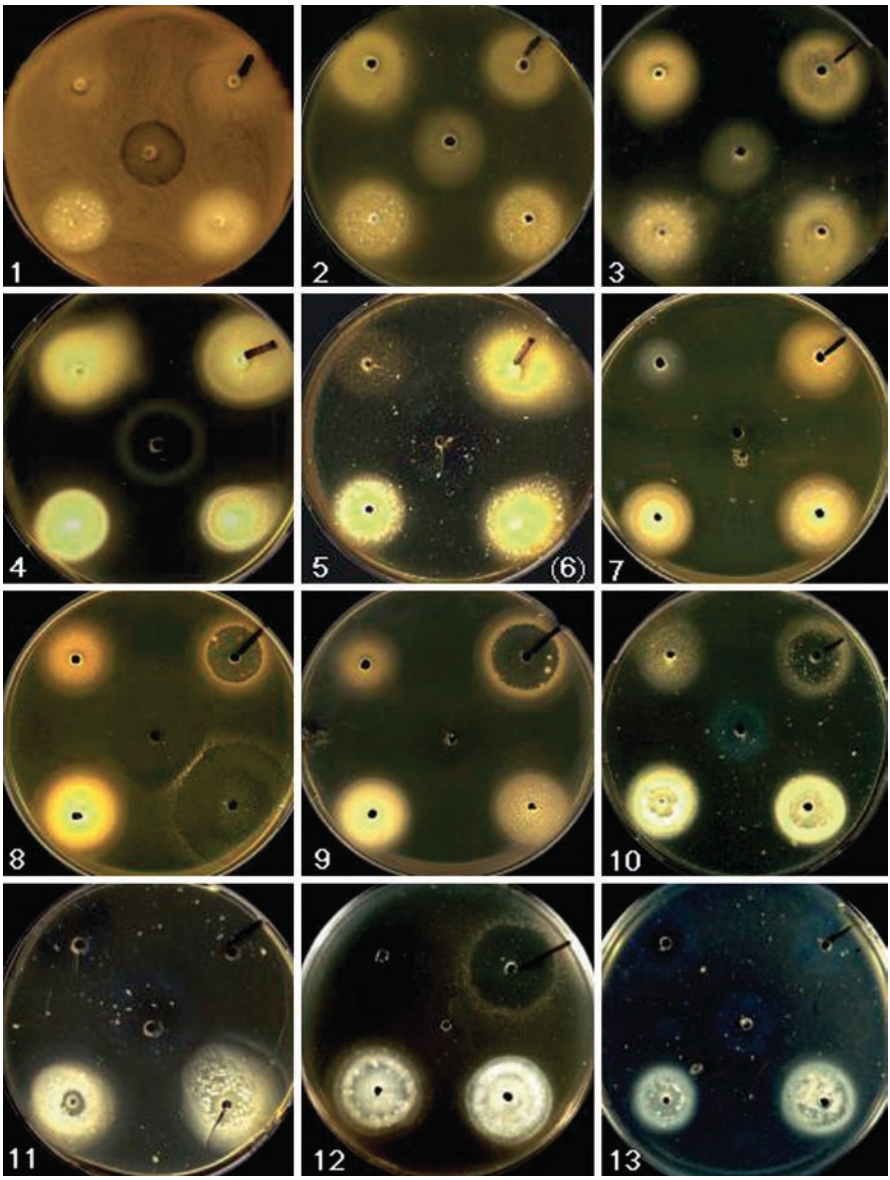


Plate 2.2 Assimilation pattern of *Malassezia* species Tween 20 (with a mark), 40, 60, 80 and Cremophor EL supplementation. *1 M. pachydermatis* (wild isolate from dog); *2 M. furfur*; *3 M. yamatoensis*; *4 M. sympodialis*; *5 M. caprae*; *6 M. dermatis* (not shown); *7 M. slooffiae*; *8 M. japonica*; *9 M. nana*; *10 M. equina*; *11 M. obtusa*; *12 M. globosa*; *13 M. restricta*. The 13 species are arranged from the left to the right according to their increasing lipid requirements

temperature is around 32–34°C; thus, both characteristics seem sufficient to preclude their presence in the environment. Surprisingly, the two fastidious species *M. globosa* and *M. restricta* have been identified by PCR; unfortunately, however, they were not obtained in culture from substrates, such as nematodes, in forest soils in Germany [12], sand stone beneath a crustose lichen in Norway [13], soils of Antarctica Dry Valleys [14], and even from methane hydrate-bearing deep-sea marine sediments in he South China sea [15].

2.1.1.1 Isolation

Below we describe the methods and media used to isolate *Malassezia* yeasts.

2.1.1.1.1 Methods

The samples, collected from skin, scalp, nails, hair, blood, catheter, or any other human or animal source, are transferred as soon as possible onto one or the other selective media to avoid dehydration of the yeasts. During transportation, moisture must be maintained as high as possible, using for instance a plastic bag or box. The samples, distributed onto the selective media in 9 cm Petri dishes, are incubated in a moist environment at 32–34°C, for at least 2 weeks.

2.1.1.1.2 Selective Media

a) *Sabouraud agar plus olive oil:* Mix 20 g glucose, 10 g bacteriological peptone and 10 mL virgin olive oil, 0.5 g chloramphenicol, 0.5 g cycloheximide in 1 L of demineralised water, adjust pH to 6.0, and add 12–15 g agar. Heat to dissolve the agar. Sterilize by autoclaving at 120°C for 15 min and aliquot as required. Addition of other oils or fatty acids, such as oleic acid, can be tested using the same recipe.
b) *Dixon agar (DA):* Mix 60 g malt extract, 20 g dessicated ox bile (Oxgall, BD Difco), 10 mL Tween 40, 2.5 g glycerol monooleate, 0.5 g chloramphenicol, 0.5 g cycloheximide in 1 L of demineralised water, adjust the pH to 6.0, and add 12–15 g agar. Sterilize by autoclaving at 115°C for 15 min, and aliquot as required.
c) *Modified Dixon agar (mDA):* Mix 36 g malt extract, 10 g bacteriological peptone, 20 g dessicated ox bile, 10 mL Tween 40, 2 mL glycerol, 2 g oleic acid, 0.5 g chloramphenicol, 0.5 g cycloheximide in 1 L of demineralised water, adjust the pH to 6.0, and add 12–15 g agar. Dissolve the agar by heating and sterilize by autoclaving at 115°C for 15 min, and aliquot as convenient.
d) *Leeming and Notman agar (LNA):* Mix 10 g bacteriological peptone (Oxoid), 0.1 g yeast extract, 5 g glucose, 8 g dessicated ox bile, 1 mL glycerol, 0.5 g glycerol monostearate, 0.5 g Tween 60, 10 mL whole fat cow milk, 0.5 g chloramphenicol, 0.5 g cycloheximide in 1 L of demineralised water, adjust the pH to 6.0, and add 12–15 g of agar. Sterilize by autoclaving at 110°C for 15 min, and aliquot as convenient.

2.1.1.1.3 Remarks

1) For an exhaustive survey, the samples, either from humans or animals, must be inoculated only onto a selective complex medium. Indeed, *M. globosa, M. obtusa,* and *M. restricta* are highly lipid-dependent, and a few isolates of primary cultures of *M. pachydermatis* do not grow on Sabouraud agar [16]. In clinical practice, the Sabouraud agar supplemented with olive oil, which can be prepared easily and rapidly, is not recommended because only *M. furfur*, *M. pachydermatis* and *M. yamatoensis* grow well on this medium [17].
2) Clinicians are also used to incubating *Malassezia* yeasts at 37°C, as this temperature is considered selective for pathogenic microorganisms. These yeasts, however, belong to the cutaneous mycobiota, and thus are ecologically adapted to a lower temperature. Because *M. globosa, M. obtusa,* and *M. restricta,* and also *M. caprae* and *M. equina,* which originated from animals, have a maximum growth temperature at 37°C [17, 18], the incubation temperature should never exceed 35°C, with an optimum between 32 and 34°C. *Malassezia* yeasts do not survive temperatures below 28°C very long, so, materials obtained from collects must not be maintained in a refrigerator before culturing. Use of a high incubation temperature and the utilization of olive oil, which does not allow the growth of most species, may explain why the knowledge of the genus remained limited to a few species for so long.
3) For epidemiological surveys, cultures must be made onto Petri dishes rather than tubes, because the latter do not allow a good separation of colonies. In the same way, the dark Dixon agars facilitate visualization of any mixed growth of *Malassezia* species, or any skin sample contaminated by other micro-organisms, such as bacteria or *Candida* spp.
4) For surveys of *Malassezia* spp. on animals, it is recommended to double the concentration of antibiotics and to use only selective media, because animal fur or/and skin are covered by a large quantity of micro-organisms. Besides, some isolates of *M. pachydermatis* have been shown to be lipid-dependent [16], and it is now well recognized that the veterinary *Malassezia* mycobiota are no longer limited to this unique species.

2.1.1.2 Maintenance of Cultures

Purified *Malassezia* isolates can be maintained on slant cultures in an incubator with a moist environment between 30 and 32°C. Cultures do not survive at room temperature very long. In routine work, they must be transferred on fresh medium every two months, but this may be one month for *M. obtusa* and *M. restricta*.

With the exception of the fastidious species *M. globosa*, *M. obtusa* and *M. restricta*, the other species can be preserved by lyophilisation. Probably, all species may survive freezing at −80°C ([19], Guého unpublished data).

a) *Lyophilization*: Cells of 4–5-day-old cultures of *Malassezia* spp. are suspended in liquid Dixon medium supplemented with 15% glycerol, and lyophilizates are stored in a refrigerator at 4°C.

b) *Freezing −80°C:* Cells of 4–5-day-old cultures of all *Malassezia* spp. are suspended in liquid mDixon medium supplemented with 15% of glycerol and aliquoted by 1 mL into 2 mL freezing Eppendorf tubes. Where possible, tubes are cooled down by −1°C per min in a progressive freezer up to −80°C, and are then stored at −80°C. To revive the yeasts, tubes are melted in a 37°C water bath, centrifuged to eliminate the medium, and subcultured by spreading the cells sparsely onto DA, mDA or mLNA medium.
c) *Liquid nitrogen:* Maintenance in liquid nitrogen was found to be the most satisfactory method of preservation for *M. pachydermatis* [20]. Therefore, it may be interesting to investigate this method of preservation for the lipid-dependent species as well.

2.1.2 Identification of *Malassezia* Yeasts Using Routine Laboratory Methods

After the species had been recognized by means of rRNA sequencing [21], it became possible to recognize their morphological, biochemical and physiological characteristics [22–26]. The subsequent description of an additional 6 species (*M. caprae, M. dermatis, M. equina, M. japonica, M. nana,* and *M. yamatoensis*) gave the opportunity to update this protocol [17], which now includes the characterization of urease -, catalase -, and β-glucosidase activities, growth at 37°C and 40°C, and the capability to grow with five water soluble lipid supplements, namely Tweens 20, 40, 60, 80 and Cremophor EL (castor oil).

2.1.2.1 Characterization of Urease Activity Using Bacto Urea Broth

Malassezia yeasts belong to Basidiomycota (see Chap. 2.2) which, in contrast to Ascomycota, are capable of hydrolysing urea. With *Malassezia* yeasts, this test is not used to separate species but rather to eliminate cultures that are contaminated by bacteria or ascomycetous yeasts, such as *Candida* spp. which are quite common on the skin. All *Malassezia* yeasts give a positive staining diazonium blue B reaction (DBB) [17, 27], but the urease test is easier to perform and provides more reliable information. The DBB staining reaction is described, with all other laboratory techniques, in the 5th edition of "The Yeasts, a taxonomic study" [28].

2.1.2.1.1 Method

A loopful of cells from 4- to 5-day-old cultures are suspended in urea broth and incubated at 37°C, irrespective of whether the yeast can grow at this temperature. The urease expression gives a bright pink to violet coloration. The reaction can be read after 1–4 h of incubation, or after 24 h in case of a doubtful reading. Any isolate giving a yellow color can be eliminated, or must be further purified if possible.

2.1.2.1.2
Medium

Difco Bacto Urea R Broth is dissolved in sterile demineralised water and aliquoted aseptically into 0.5 mL volumes in tubes. The tubes can be stored in a freezer for up to 6 months. The ready-made urea-indole medium (bioMérieux) can be used as well.

2.1.2.1.3
Remark

The reaction may give a doubtful reading on solid medium, such as Christensen's urea agar (numerous strains of *M. pachydermatis* missing an urease activity on this medium were observed by A. Velegraki, unpublished data).

2.1.2.2
Characterization of the Catalase Activity Using Hydrogen Peroxide

This test is commonly used in bacteriology as a first step in identification. It appeared to be useful within the genus *Malassezia* as well.

2.1.2.2.1
Method

Catalase activity of *Malassezia* yeasts is determined by adding a drop of hydrogen peroxide onto a culture smear on a glass slide or in a small seeded glass tube. The enzyme catalyzes the decomposition of peroxides formed during oxidation reactions. A positive result is indicated by effervescence, caused by the liberation of free oxygen. It is recommended to use only glass for this test in order to avoid doubtful results.

2.1.2.2.2
Reagent

The test is performed using 10–20% (vol. instead P% in France) hydrogen peroxide or the commercial reagent ID color Catalase (bioMérieux), which makes the reaction easier to read and more stable, owing to the presence of a thickener.

2.1.2.3
Characterization of β-Glucosidase Activity Using the Esculin Medium

Certain *Malassezia* species possess a β-glucosidase that is able to hydrolyse the glucosidic bond of esculin, thus liberating glucose and esculetin. The phenol moiety reacts with the iron to give a black color.

2.1.2.3.1
Method

The esculin medium in tubes is inoculated by stabbing the yeast culture centrally into the medium using a platinum wire, and is incubated at 37°C, irrespective of whether the yeast can grow at this temperature. There is no need to screw the cap down tightly. Examine daily for 5 days. A positive reaction is indicated by blackening of the medium, whereas absence of blackening indicates lack of β-glucosidase activity.

2.1.2.3.2
Media

Esculin agar (EA) Mix 10 g bacteriological peptone, 1 g ferric ammonium citrate, 1 g esculin per 1 L demineralised water, adjust the pH to 7.4, and add 15 g agar. Dissolve by heating and distribute in 6 mL volumes in tubes. Sterilize by autoclaving at 115°C for 15 min. Store at 2–6°C for 2 years.

2.1.2.3.3
Remarks

1) The esculin medium may be used directly in Petri dishes, but then the reaction can be slower, thus resulting in doubtful answers.
2) The ready-made Esculin Iron Agar (esculin 0.1 g, ferric ammonium citrate 0.5 g, agar 15 g, demineralised water 1 l) (Fluka) can been used as well, but with addition of 1% peptone. Furthermore, the esculin concentration is lower in this latter medium, and such differences in the composition of esculin media can explain discrepancies in results obtained.
3) Japanese authors [29, 30] have combined the esculin test to a growth medium (Tween 60-esculin agar), but this ready-made medium does not allow good reading of the β-glucosidase activity and may also increase the cost of identification.
4) Commercial bacteriological esculin tubes (bio-Rad) can be used as well, but may increase the cost.

2.1.2.4
Growth at 37°C and 40°C

Subcultures are incubated using one or the other selective medium at 37 and 40°C to get supplementary key characteristics.

2.1.2.4.1
Remark

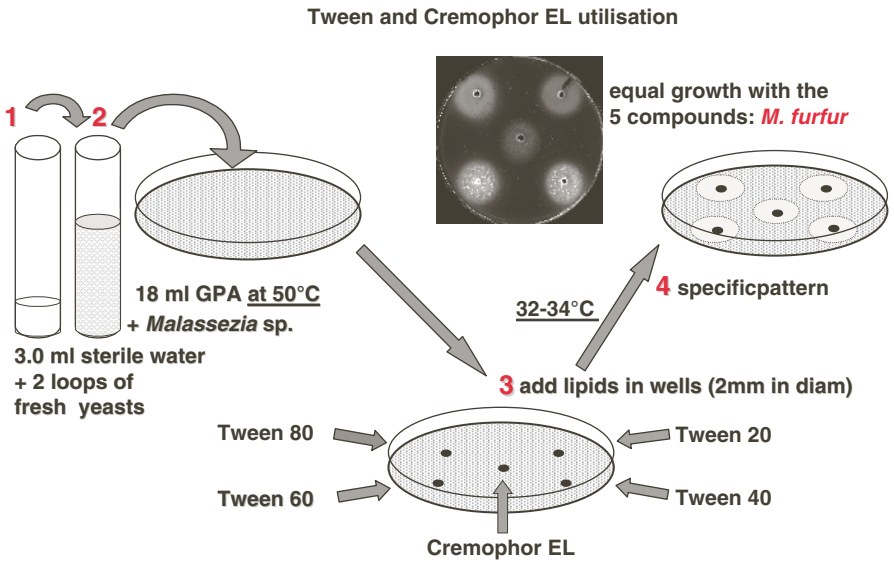
Growth at 37°C may give doubtful responses, since several species are limited to this temperature, as indicated above.

2.1.2.5 Utilization of Tweens 20, 40, 60, 80, and Cremophor EL (see Scheme 2.1 and Plates 2.1 and 2.2)

Lipophilic and lipid-dependent *Malassezia* yeasts require complex media that are enriched with lipids. These particular requirements were found to be useful to separate species [25, 26]. Strains are tested for their capacity to grow on Sabouraud agar (GPA), separately supplemented with Tweens 20, 40, 60, 80, and Cremophor EL (CrEL or castor oil) as an unique lipid source. These five water-soluble compounds can be tested together, using their capacity to diffuse into a solid basic medium. Many insoluble lipids have been tested, but so far without improving the identification.

2.1.2.5.1 Methods

Two loops of a 4–5-day-old *Malassezia* culture are suspended in 3.0 mL of sterile demineralised water. This inoculum is added to 18 mL of a molten Sabouraud agar maintained at 50°C, and the mixture is poured immediately in a 9-cm Petri dish (Scheme 2.1). After complete solidification, wells are made with a 2-mm diameter punch, 4 devoted to test the growth using Tweens 20, 40, 60 and 80 clockwise around, with a mark to indicate the position of Tween 20, and a fifth hole in the center to test growth with Cremophor EL. The wells are filled with approximately 15 µL of each product (Sigma), which are not sterilized but aliquoted in 2 mL Eppendorf tubes. The dishes are incubated for 7–10 days (for good pictures) at 32–34°C in a moist environment, and turned upside down on the second day to delay their dehydration.



Scheme 2.1 Method to evaluate growth of *Malassezia* spp. with the five individual lipid supplements

2.1.2.5.2 Medium

a) *Glucose-peptone agar (GPA) (also named Sabouraud agar (SA)).* Mix 20 g glucose and 10 g bacteriological peptone in 1 L of demineralised water, adjust the pH to 6.0, and add 18 g agar. Heat to dissolve the agar, distribute in 18 mL-volumes in tubes and sterilize by autoclaving at 120°C for 15 min.

2.1.2.5.3 Remarks

1) To avoid any contamination, 0.5 g cycloheximide per L can be maintained in SA. The assimilation patterns can be scanned, but then the wells are filled with Tween supplements counter-clockwise. For pictures, always add a black sheet as bottom or cover, when camera or scanner are used, respectively.
2) Using the diffusion method, each soluble lipid supplement gives a concentration gradient, which, consequently, may provide supplementary information. Indeed, growth can start close to the well with a high concentration of the supplement, thus giving a full disk of colonies (i.e., *M. furfur, M. slooffiae*) (Scheme 2.1, Plates 2.1-2, 7 and 2.2-2, 7). In contrast, growth may appear mainly at some distance from the well after dilution of the supplement, resulting in a ring of colonies (i.e., Tween 20 of *M. japonica,* the type strain of *M. nana,* and *M. equina*) (Plates 2.1 and 2.2. 8, 9 and 10, respectively). Growth can also start at some distance from the well after diffusion of the supplement, but with secondary growth progression back towards the well, resulting in complete or incomplete centripetal growth (i.e., Tween 20 of *M. caprae*, *M. dermatis*, *M. sympodialis,* or *M. yamatoensis*; see also Tween 20 of *M. sympodialis* in Fig. 2.4f). Then supplements can generate a neat inhibition area, and later, a secondary growth zone within this area (i.e., Tween 20, 40, and Cr EL of *M. pachydermatis* or Tween 40 of *M. japonica*) (Fig. 2.1f, Plates 2.1-1, 8 and 2.2-1, 8).
3) Since the first description of a practical method to identify *Malassezia* yeasts [25], several improvements have been suggested, including assimilation of Cremophor EL and characterization of β-glucosidase activity [17, 22, 24, 26, 31]. However, the addition of six new species (viz. *M. caprae, M. dermatis, M. equina, M. nana*, *M. japonica* and *M. yamatoensis*) resulted in similar physiological patterns of several *Malassezia* species, and thus in a doubtful identification, e.g., of isolates belonging to *M. caprae* and *M. nana* [17]. In these cases, the identification should be confirmed by sequence analysis of the D1/D2 domains of the LSU rRNA gene and/or the ITS1+2 regions, in order to reduce the risk of misidentifications (see Chap. 3.1). Furthermore, attention should be given to the source of such isolates, whether human or animal, as well as their precise location on them.
4) The Japanese system of identification [29, 30] requires, after the catalase test with 3% hydrogen peroxide has been performed, subculturing of the isolate on several expensive media (CHROMagar-*Malassezia*, SDA, Cremophor EL agar, Tween 60-esculin Agar). Moreover, this system needs to be evaluated with all currently known species, and additional isolates from various sources for each of them.

2.1.3 Biodiversity of *Malassezia* Yeasts

Characteristics of all species have been re-evaluated for the 5th edition of "The yeasts: a taxonomic study" [17]. In this book, species are systematically arranged alphabetically [32]. In this chapter, we list them according to their decreasing biochemical and physiological capabilities as shown in Plates 2.1 and 2.2. This option somewhat parallels the chronological order of discovery, since the oldest described species are not too demanding in their grow requirements (i.e., *M. furfur, M. pachydermatis* and *M. sympodialis*). Urease and DBB staining reactions are positive for all species, and thus these characteristics are not further included. Similarly, all species can be considered to have a co-enzyme Q system with nine isoprenologs, i.e., CoQ-9 [33], even though this has not yet been investigated for all species [17].

2.1.3.1 *Malassezia pachydermatis* (Weidman) Dodge (1925)

This species, isolated for the first time by Weidman in 1925 [35] from a captive Indian rhinoceros, was described as *Pityrosporum pachydermatis* and transferred to the genus *Malassezia* by Dodge in 1935 [34]. In contrast to similar organisms observed in scales of PV or pityriasis capitis, *M. pachydermatis* was able to grow on regular rich media. Unfortunately, the original strain isolated by Weidman was not preserved. In 1955 Gustafson described *Pityrosporum canis* from the ear of a healthy dog [36]. For years, its synonymy with the previously described species *P. pachydermatis* remained uncertain, but molecular approaches, such as analysis of mol% G+C, nuclear DNA/DNA reassociation experiments, and rRNA or rDNA sequence comparisons, applied to isolates from various sources, in particular, five captive Indian rhinoceros, allowed to validate the species name *M. pachydermatis* [37]. However, the species was also found to be genetically heterogeneous and in the course of evolution, probably due to adaptation to their hosts [38, 39].

2.1.3.1.1 Neotype Strain

CBS 1879, isolated from otitis externa in dog. Because the original culture of *Pityrosporum pachydermatis* [35] has not been preserved, the strain CBS 1879, which had been deposited as the type strain of *Pityrosporum canis* [36], was selected as neotype of *M. pachydermatis,* when it was demonstrated that both old names were synonyms [23, 37].

2.1.3.1.2 Morphological Characteristics

After growth at 32°C on mDA on SA for 7 days, single colonies (Fig. 2.1a) are convex, 4–5 mm in diameter, butyrous to brittle, somewhat shiny, pale yellowish-cream, and with an entire, straight or somewhat undulating margin. A few isolates from dogs were found to

be lipid-dependent with smaller colonies [16], and belonged to the same sequevar d [21, 38]. An unusual pink isolate from civet (GM 439) clustered as sequevar a with strains from dogs, including neotype strain CBS 1879, and all strains from humans [21]. Cells are ellipsoidal to

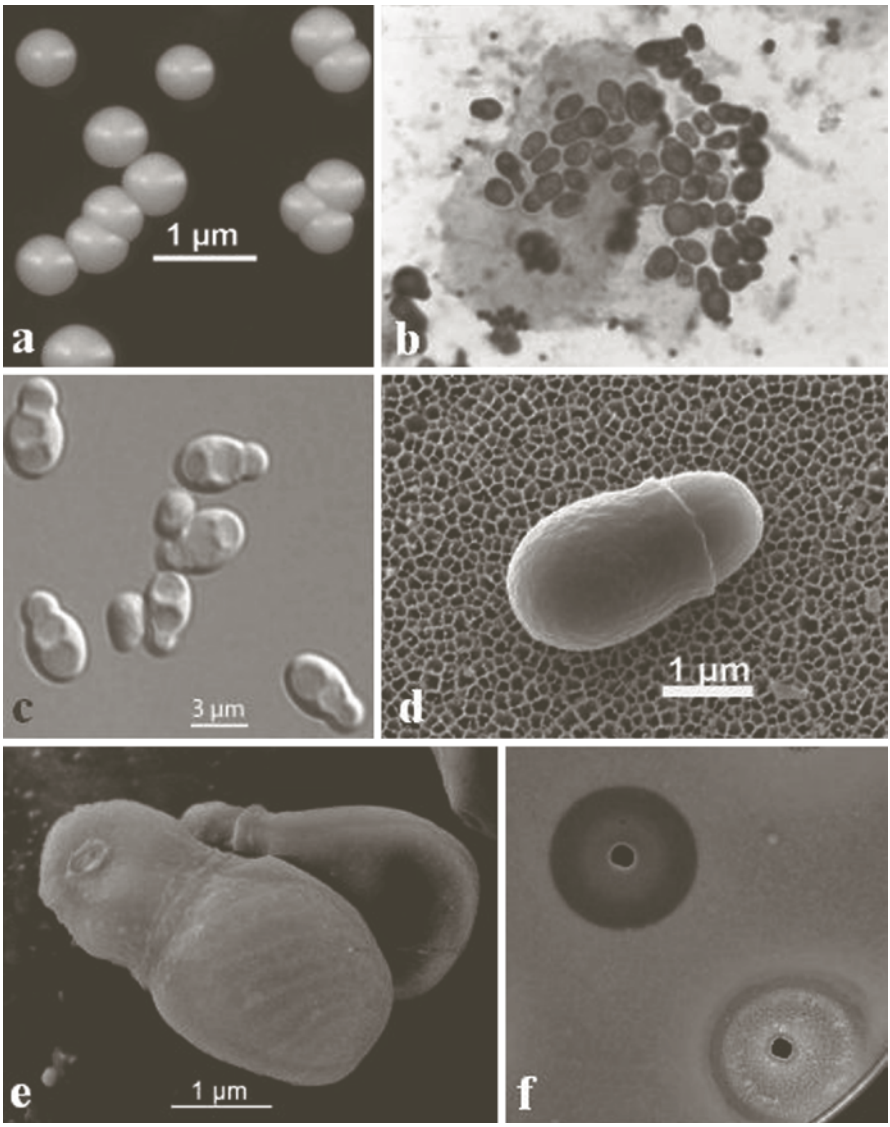


Fig. 2.1 *M. pachydermatis*. (**a**) Convex colonies with an entire margin; (**b**) ovoid Gram stained yeasts in dog ear cerumen (picture by the courtesy of Guillot); (**c**–**e**), Nomarski's and SEM micrographs showing ovoid to short cylindrical yeast cells with a broad budding site; (**e**), notice the helicoidal structure of the cell wall; (**f**), details of Cr EL and Tween 40 utilization showing secondary growth within the inhibitory areas

short cylindrical, 4–5 × 2–2.5 μm, with monopolar budding on a broad base (Fig. 2.1b–e). Filaments were never observed in this species.

2.1.3.1.3
Physiological and Biochemical Characteristics

All isolates grow at 37 and 40°C. Differences in catalase and β-glucodidase expression, and Tweens 20, 40, 60, 80 and Cremophor EL (CrEL) growth reactions occur in all rDNA genotypes. For instance, the neotype strain CBS 1879 presents growth inhibition with Tweens 20, 40 and CrEL (Fig. 2.1f and Plate 2.1-1), with secondary growth within this inhibitory area after diffusion of these supplements [40]. Strains from dogs display three sequevar types, namely sequevar a, including the type strain and all human isolates, and sequevars d and e. With the latter type, only CrEL shows an inhibitory area (Plate 2.1-1). Other isolates may be not inhibited by any of the five supplements, but more experiments are needed to determine whether or not, these differences are stable strain characteristics.

2.1.3.1.4
Ecology

Malassezia pachydermatis is a lipophilic, but not a highly lipid-dependent species [3, 41, 42]. The species is most often associated with animals, particularly ears, and/or healthy or lesional skin of canines, but has also been isolated from numerous other animals [43, 44]. Its prevalence in rhinoceroses was confirmed several times [21, 37, 45, 46], but is probably largely underestimated in animals other than cats, dogs and rhinoceroses (see Chaps. 3.3 and 10). *M. pachydermatis* can be isolated from human blood and sputum, and is implicated in infections of neonates, under parenteral alimentation enriched by lipids. However, its presence on humans is transitory and the source of these infections may be linked to pet animals [47–49].

2.1.3.2
Malassezia furfur (Robin) Baillon (1889)

Because neotype cultures corresponding to the old names *Malassezia furfur* and *P. ovale* proved to be synonymous, the former name is maintained as the generic type species [23].

2.1.3.2.1
Neotype Strain

The strain CBS 7019, isolated from PV scales on the trunk of a 15-year-old girl in Finland, is considered as the neotype strain of this species. CBS 1878, however, has also been designated as neotype, but this strain originally represented *P. ovale* (Bizzozero) Castellani and Chalmers [50], a fungus that was regularly seen in scales of pityriasis capitis.

2.1.3.2.2 Morphological Characteristics

After 7 days at 32°C on mDA, single colonies (Fig. 2.2a) are 4–5 mm in diameter, dull, umbonate or slightly folded, butyrous to friable, smooth, with a convex elevation and an entire to slightly lobate margin. Colony texture is soft and the cells are easy to emulsify.

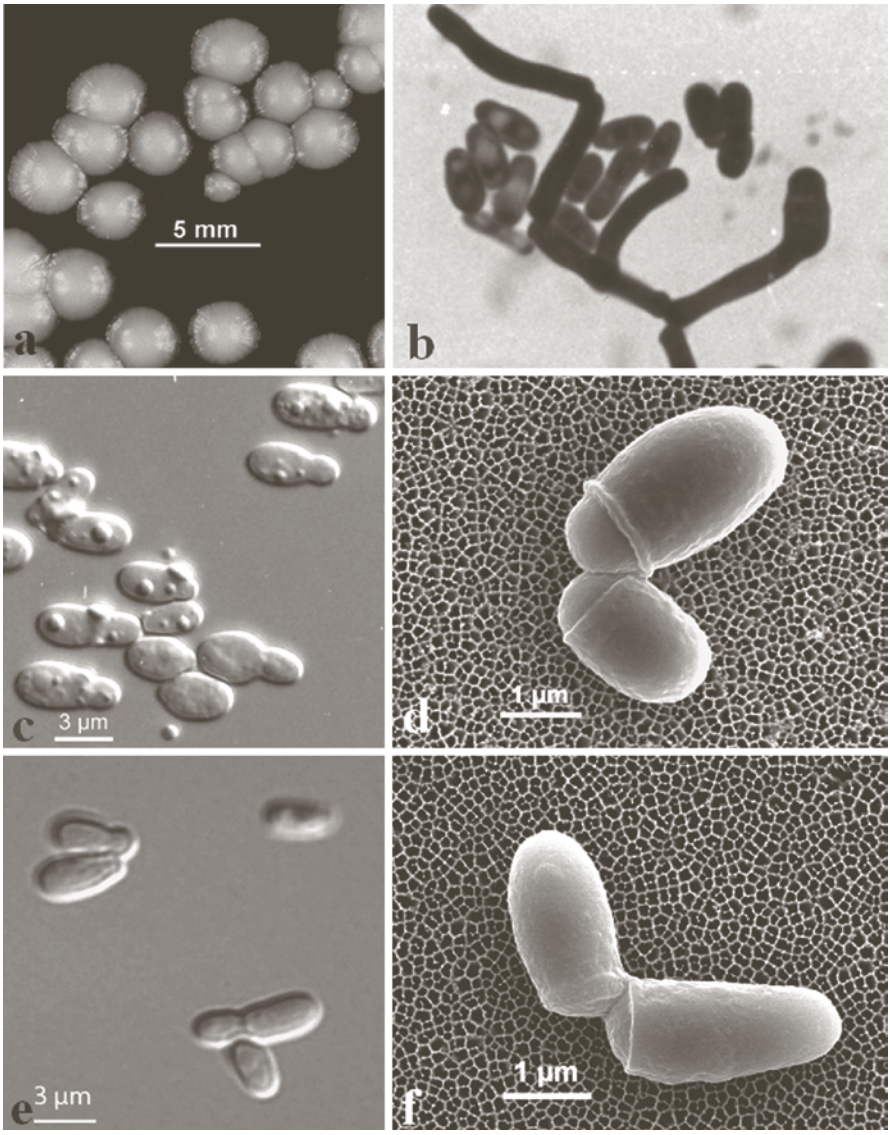


Fig. 2.2 *M. furfur.* (**a**) Umbonate colonies with a slightly lobate margin; (**b**) Gram stained ellipsoidal yeasts and pseudohyphae of a spontaneously filamentous strain; (**c**–**d**), Nomarski's and SEM micrographs, respectively showing the ellipsoidal yeasts of CBS 7019; (**e**–**f**), Nomarski's and SEM micrographs, respectively showing the cylindrical yeasts of CBS 1878

Cells are variable in size and shape, cylindrical to ovoid, 2.5–8 × 1.5–3 µm (Fig. 2.2b–f), or globose, 2.5–5 µm in diameter, with percurrent budding on a more or less broad base. Pseudohyphae may be occasionally produced in some cultures (Fig. 2.2b) and, if present, this feature seems to be a stable strain character. Pseudohyphae may also occur after culturing under appropriate medium conditions (see Chap. 2.3).

2.1.3.2.3
Physiological and Biochemical Characteristics

Malassezia furfur can routinely be identified by its capacity to grow up to 41°C, a strong catalase reaction, a more or less marked β-glucosidase activity (but in contrast to *M. sympodialis* this never turns very dark after 24 h incubation at 37°C), and shows more or less equal growth in the presence of four Tweens and CrEL (Note: growth for the latter supplement may be somewhat weaker) as sole sources of lipid (Scheme 2.1, Plates 2.1-2 and 2.2-2). *M. furfur* has an essential requirement for olive oil or oleic acid for growth on malt or Sabouraud agars, but the species is only mildly lipid-dependent, as any lipid supplement is sufficient for its growth [22]. Furthermore, all lipid supplements used for *Malassezia* identification are assimilated similarly. On the contrary, only a few species are able to grow well with oleic acid or olive oil as lipid supplementation, namely *M. furfur* and *M. pachydermatis* and, to a lesser extent, *M. japonica* and *M. yamatoensis* [17]. Optimum temperature for growth is near 34°C, but good growth occurs at 37°C, and the maximum temperature for growth is 41°C. Species-specific characteristics are rather stable for this species. However, a few atypical variants may occur, such as isolates that grow well with Tween 80 only [51] or fail to grow with CrEL [8, 52]. These atypical isolates need further studies with molecular methods. *M. furfur* is one of the most robust lipid dependent species, and its growth is merely induced by an amino nitrogen in combination with a lipid source [26]. In contrast to *M. globosa, M. obtusa, M. restricta, M. slooffiae* and *M. sympodialis, M. furfur* is able to utilize glycine as nitrogen source [53]. Salkin and Gordon [54] examined fresh isolates of *Malassezia* (reported as *Pityrosporum*) species with globose, ovoid to cylindrial cells and different fatty acid requirements, and they suggested that *P. ovale* and *P. orbiculare* were both synonymous with *M. furfur*. However, in this pre-DNA era it was impossible for the authors to recognize that they were dealing with different taxa. This proposed synonymy was maintained by Yarrow and Ahearn [55]. Unfortunately, the original type material and isolates designated as *P. orbiculare* on the basis of cell shape and inability to grow on oleic acid were not preserved. Therefore, in the taxonomic revision [23], it was proposed to consider *P. orbiculare* as a doubtful species, which may represent a probable synonym of *M. globosa* and not *M. furfur*.

2.1.3.2.4
Ecology

Malassezia furfur is known from various hosts and body sites. In humans, it has been isolated from scalp, face, dandruff, arms, legs, urine, blood, hair, nails, eyes, and the nasal cavity. The high temperature tolerance of *M. furfur*, contrary to *M. globosa*, could explain

why *M. furfur* is more frequently isolated from pityriasis versicolor (PV) in warmer climates [17, 48]. The species has also been detected from a hospital floor [56], and occasionally from cats [57, 58], dogs [59], horses [60], cows, asymptomatic or with otitis [61, 62], and bats [63]. However, more veterinary surveys will be necessary to fully evaluate the prevalence of *Malassezia* lipid-dependent species on animals.

2.1.3.3
Malassezia yamatoensis Sugita, Tajima, Takashima, Amaya, Saito, Tsuboi & Nishikawa (2004)

2.1.3.3.1
Type Strain

CBS 9725 (JCM 12262), isolated from a nose lesion of a seborrheic patient [64].

2.1.3.3.2
Morphological Characteristics

After 7 days at 32°C on mDA, single colonies (Fig. 2.3a) are flat to convex, 3–4 mm in diameter, butyrous, shiny, pale yellowish-cream, smooth, and with an entire, somewhat

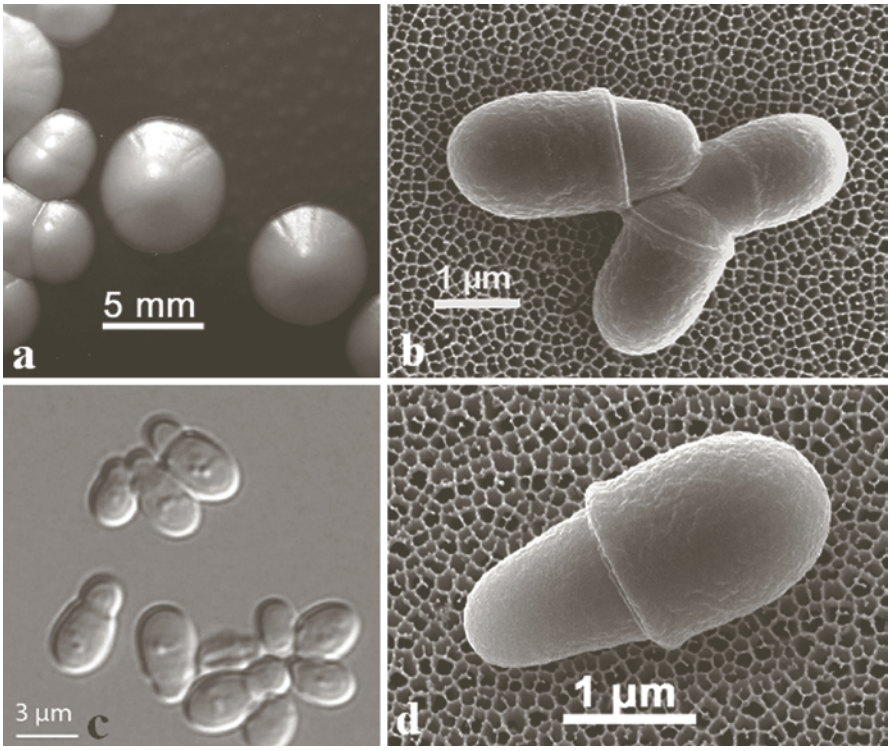


Fig. 2.3 *M. yamatoensis.* (**a**) Convex colonies with a slightly folded and undulate margin; (**b**–**d**) Nomarski's and SEM micrographs showing short cylindrical yeasts and their broad budding sites

undulating margin. Cells are ovoid to short cylindrical, 3–4 × 2.4–3 µm, with monopolar budding on a broad base (Fig. 2.3b–d).

2.1.3.3.3
Physiological and Biochemical Characteristics

Malassezia yamatoensis can be identified by its capacity to grow at 37°C, but not at 40°C, a strong catalase reaction and lack of β-glucosidase activity. These characteristics distinguish the species from *M. sympodialis*. Equal growth that progresses centripetally appears in the presence of all four Tweens, and a more or less marked growth occurs with CrEL (Plates 2.1-3 and 2.2-3).

2.1.3.3.4
Ecology

Malassezia yamatoensis seems to be a rare species, which has been reported from humans with atopic or seborrheic dermatitis, and more rarely from healthy individuals [64].

2.1.3.4
Malassezia sympodialis Simmons & Guého (1990)

2.1.3.4.1
Type Strain

CBS 7222, isolated from the auditory tract of a healthy 33-year-old male [65]. *M. sympodialis* corresponds to *M. furfur* serovar A as previously recognized [66].

2.1.3.4.2
Morphological Characteristics

After 7 days at 32°C, single colonies (Fig. 2.4a) are flat to somewhat elevated in the center, approximately 6–8 mm in diameter, pale cream to yellowish-brown, shiny, smooth, butyrous, and with an entire or finely folded margin. The cells are ovoid to globose, 2.5–4.0 x 1.5-3.5 µm (Fig. 2.4b–e), with enteroblastic, percurrent, monopolar budding, and with buds that may emerge sympodially from a relatively narrow base. Cultures of fresh isolates on mDA develop crystal precipitates in the agar, diffusing around the colonies and resembling eventual contaminations. This phenomenon is common to other species related to *M. sympodialis* [67], namely *M. dermatis, M. caprae, M. equina,* and *M. nana,* and also *M. globosa.* This precipitate in the culture medium is different from the white precipitate that usually surrounds Tween 40 and 60 in the testing dishes, even in the absence of growth (Plate 2.1 and 2.2). There are conflicting reports on the ability of the species to form filaments in culture. According to some workers, *M. sympodialis* is able to form filaments

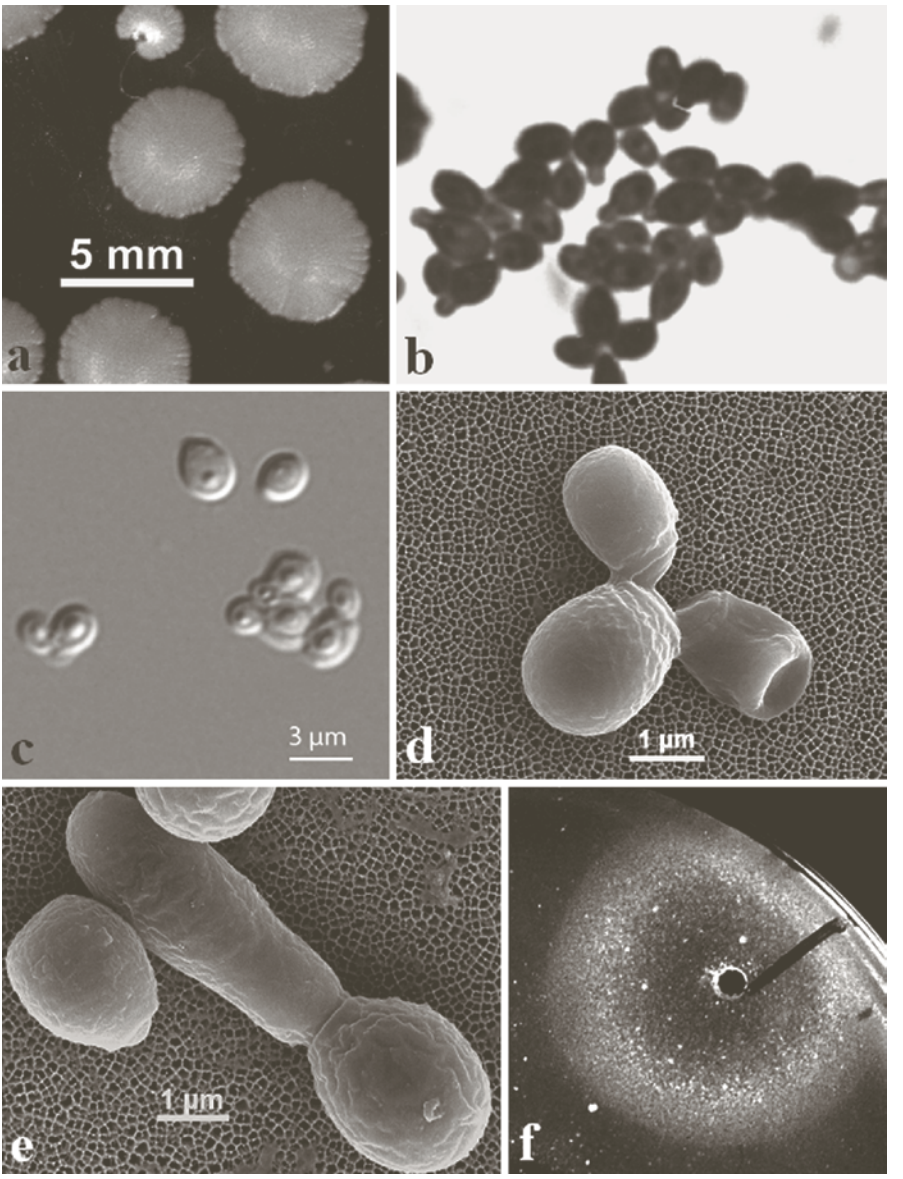


Fig. 2.4 *M. sympodialis.* (**a**) Flat colonies that are smooth and slightly elevated in the center with a finely folded margin; (**b**–**e**) Nomarski's and SEM micrographs showing ovoid to globose yeasts; (**d**) regular clover leaf configuration of cells, which is a typical feature of this species; (**e**) germination tube showing the helicoidal structure of the cell wall; (**f**) growth with Tween 20 that progresses centripetally, but is still incomplete towards the well after 4 days of incubation

in vitro on a complex medium [68]. Cultures of this species usually do not display any hyphae, although short filaments could occasionally be observed in the type culture using scanning electron microscopy (Fig. 2.4e).

2.1.3.4.3
Physiological and Biochemical Characteristics

Malassezia sympodialis can be identified by its capacity to grow at 40°C, a catalase reaction and a strong β-glucosidase activity (note: the reaction is getting dark already after 24 h incubation at 37°C), and good growth in the presence of all four Tweens, but growth is absent with CrEL (Plates 2.1-4 and 2.2-4). The disk of colonies around Tween 20 is often wider than that obtained with the three other Tweens, but growth starts at some distance from the well after diffusion of the compound and progresses centripetally, thus remaining after a few days' incubation as a zone of incomplete growth near the well (Fig. 2.4f). With CrEL, growth is usually absent, but fresh isolates can develop a ring of tiny colonies, or they show a white precipitate only, which occurs at some distance from the well (Plates 2.1-4 and 2.2-4).

2.1.3.4.4
Ecology

Malassezia sympodialis is an inhabitant of healthy human skin. In particular, it occurs on the back and chest, but also at other body areas, such as the auditory tract [65]. The species has also been isolated from human PV [21, 23, 69, 70] and occasionally from healthy feline skin [57, 71–73], goats [60], cows [61] and bats [63].

2.1.3.5
Malassezia caprae Cabañes & Boekhout (2007)

2.1.3.5.1
Type Strain

CBS 10434 (MA 383), isolated from healthy skin of goats, Barcelona, Spain [18].

2.1.3.5.2
Morphological Characteristics

After 7-10 days at 32°C on mDixon agar, single colonies (Fig. 2.5a) are small, 1–2 mm in diameter, whitish to cream-colored, smooth, glistening or dull, butyrous, and moderately convex with an entire to lobate margin. Cells are globose, somewhat rhomboidal or ovoid, 2.5–4 × 2.2–3.5 µm, with buds emerging from a narrow to moderately broad base (Fig. 2.5b–d). Hyphae have not been observed.

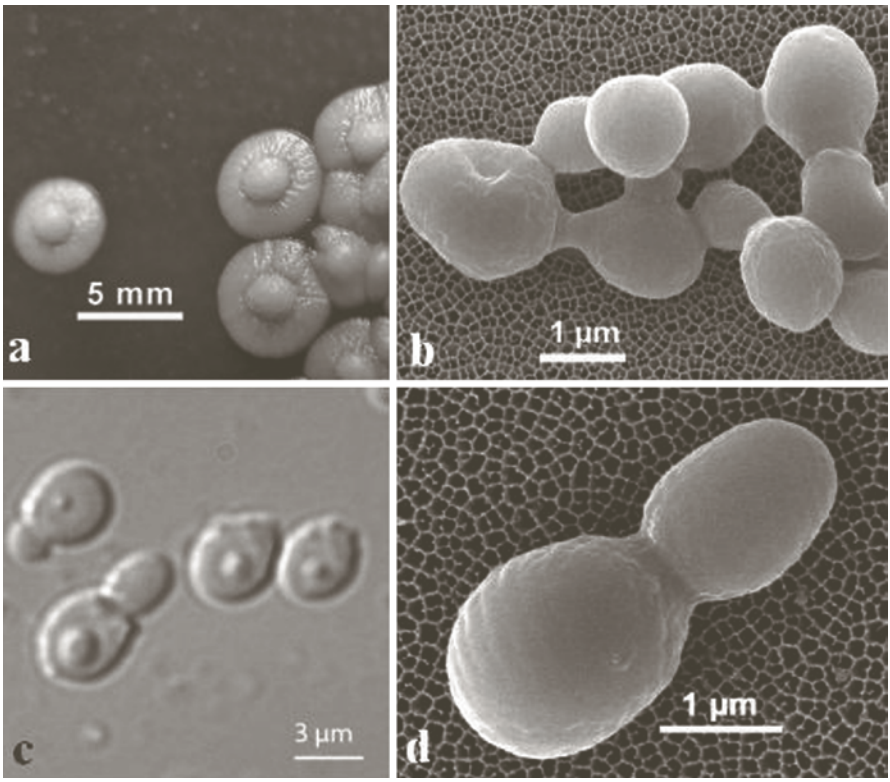


Fig. 2.5 *M. caprae.* (**a**) Convex to umbonate colonies with a finely folded to undulate margin; (**b**–**d**), Nomarski's and SEM micrographs of ovoid to slightly rhomboidal (**b**) yeasts showing a quite narrow budding site, and the corrugate helicoidal feature of the cell wall (**d**)

2.1.3.5.3
Physiological and Biochemical Characteristics

Malassezia caprae can be identified by lack of growth at 40°C, weak growth at 37°C, presence of a catalase reaction, β-glucosidase activity, and good growth in the presence of four Tweens. Growth may be somewhat weaker with Tween 80, and delayed centripetal growth with Tween 20 is observed to be similar to that of *M. sympodialis* (Plates 2.1-5 and 2.2-5). This species belongs to the cluster of species related to *M. sympodialis* [67]. It can be differentiated from the latter species by growth that is limited at 37°C. *M. slooffiae* is characterized by a weak and restricted growth with Tween 80, but the latter species, in contrast to *M. caprae,* is able to grow at 40°C and does not split esculin. *M. caprae* does not grow with CrEL, but can develop a weak precipitate with this nutriment (Plate 2.1-5).

2.1.3.5.4
Ecology

Malassezia caprae is so far only known from the healthy skin of goats and horses [18].

2.1.3.6
Malassezia dermatis Sugita, Takashima, Nishikawa & Shinoda (2002)

2.1.3.6.1
Type Strain

CBS 9169 (JCM 11348), isolated from skin lesions of a patient with atopic dermatitis, Tokyo, Japan [74].

2.1.3.6.2
Morphological Characteristics

After 7 days at 32°C on mDixon agar, single colonies (Fig. 2.6a) are 5–6 mm in diameter, flat to somewhat apiculate centrally, butyrous, shiny to dull, pale yellowish-white, and with an entire or finely folded margin. Cells are globose, ovoid or ellipsoidal, 3.8–4.8 × 2.5–

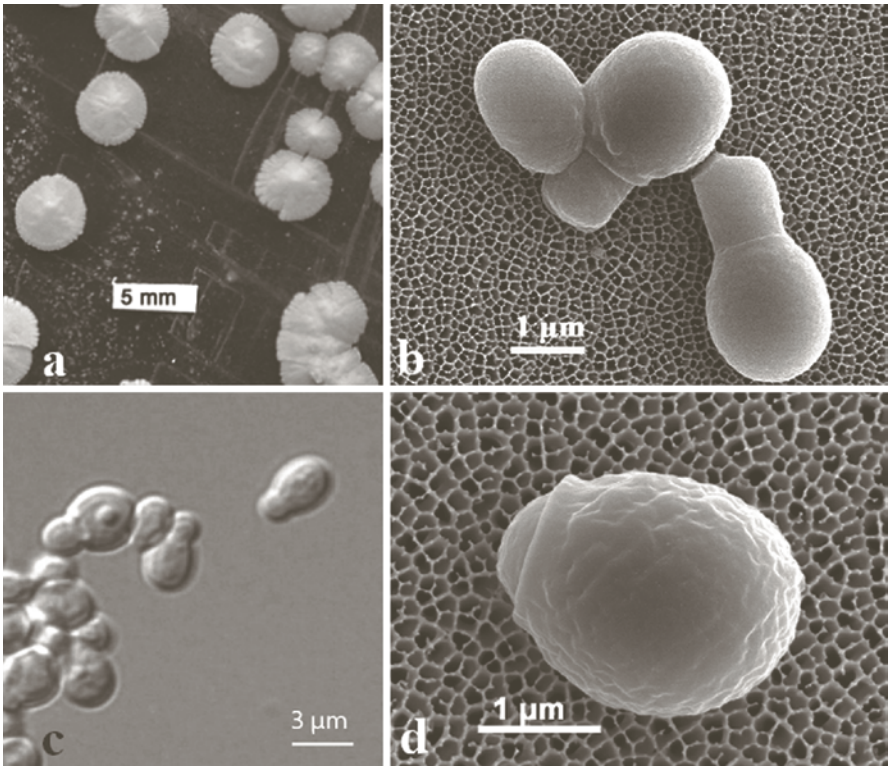


Fig. 2.6 *M. dermatis*. (**a**) Flat colonies which are slightly elevated in the center and have a folded margin (note the typical precipitate within the agar around the colonies); (**b**–**d**) Nomarski's and SEM micrographs of ovoid to globose yeasts resembling those of *M. sympodialis*. (**d**) The ovoid yeast shows a regular helicoidal feature of the cell wall

3.2 µm, with monopolar, eventually sympodial budding on a moderately broad base (Fig. 2.6b–d). The species is not known to produce any filaments.

2.1.3.6.3
Physiological and Biochemical Characteristics

Malassezia dermatis can be identified by lack of growth at 40°C, and lack of both catalase and β-glucosidase reactions. The former test appears contradictory with the original description in which it was given as positive [74], and the latter is absent in this original description but was confirmed by Kaneko et al. to be negative [29, 30].

Growth occurs with all four Tweens. Growth with Tween 80 may be weaker, similar to that of *M. caprae*, and is absent with CrEL (see *M caprae* in Plates 2.1-5 and 2.2-5). As this assimilation pattern may look similar to that of *M. sympodialis* or *M. caprae*, which may have the same delayed centripetal growth with Tween 20, the absence of the two enzymes clearly differentiate *M. dermatis* from the other two species. *M. restricta* is the only other lipid-dependent species that lacks both catalase and β-glucosidase activities, but this species grows only on complex media, including several lipid compounds.

2.1.3.6.4
Ecology

Malassezia dermatis was first isolated from skin lesions of a few patients with atopic dermatitis [74]. It has also been isolated from 19 out of 160 healthy volunteers in Korea [75]. Contrary to the reported catalase positive reaction in the two corresponding papers [74, 75], the three strains, including the type CBS 6169, examined for this work and the 5th edition of "The Yeasts, a taxonomic study" were found to have a catalase negative reaction [17]. The molecular identification is more trustable than the routine laboratory techniques, but it is important to control carefully all characteristics. Indeed a strain, growing with the four Tweens but unable to give a catalase reaction, can only be *M. dermatis*.

2.1.3.7
Malassezia slooffiae Guillot, Midgley & Guého (1996)

2.1.3.7.1
Type Strain

CBS 7956 (JG 554), isolated from skin of the ear of a pig [23].

2.1.3.7.2
Morphological Characteristics

After 7 days at 32ºC on mDixon agar, single colonies (Fig. 2.7a) are flat or somewhat raised, 3–4 mm in diameter, shiny, pale yellowish-brown, butyrous, with a somewhat

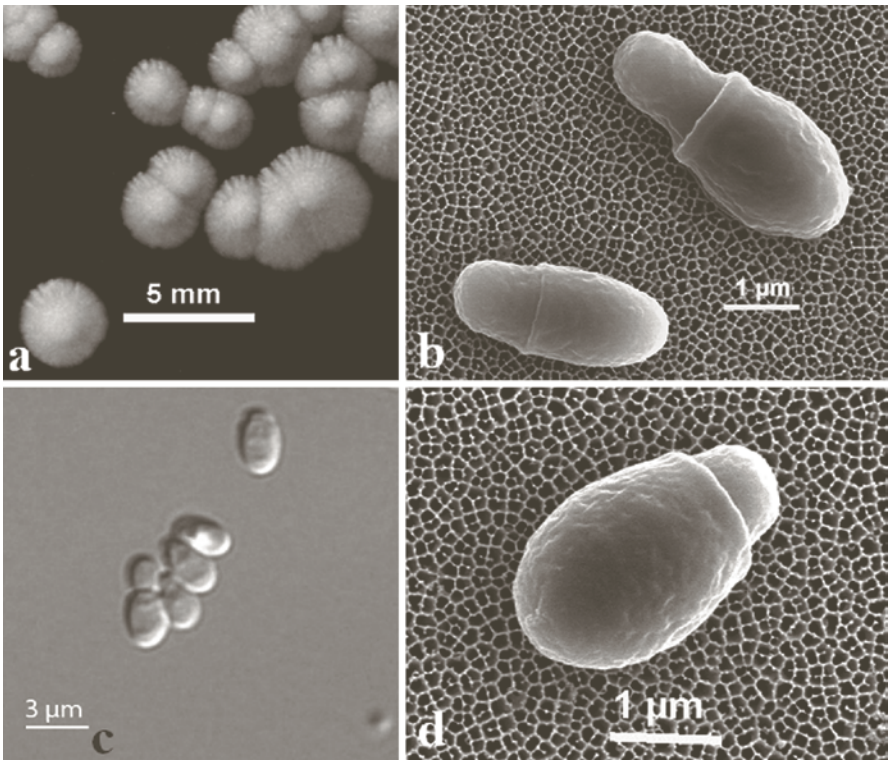


Fig. 2.7 *M. slooffiae.* (**a**) Flat to slightly apiculate colonies with a regularly folded margin; (**b**–**d**), Nomarski's and SEM micrographs of short cylindrical yeasts with a wide and more or less marked budding site

roughened surface and a finely folded margin. The cells are short, cylindrical, 1.5–4 × 1–2 µm, and budding is monopolar, percurrent and occurs on a broad base (Fig. 2.7b–d). The species is not known to produce any filaments.

2.1.3.7.3
Physiological and Biochemical Characteristics

Malassezia slooffiae can be identified by its capacity to grow at 40°C, and shows a catalase reaction, but β-glucosidase activity is absent. The key characteristic is that growth with Tween 80 is always weak and restricted in comparison to the equal growth obtained with the other three Tweens, and growth with CrEL is absent (Plates 2.1-7 and 2.2-7).

2.1.3.7.4
Ecology

The species is found in low frequency on healthy or lesioned human skin, usually in association with *M. sympodialis, M. furfur, M. globosa* or *M. restricta* [23]. *M. slooffiae* is also com-

monly isolated from animal skin, especially that of pigs [43], and also from cats [76, 77], cows [61] and goats [78].

2.1.3.8
Malassezia japonica Sugita, Takashima, Kodama, Tsuboi & Nishikawa (2003)

2.1.3.8.1
Type Strain

CBS 9431 (JCM 11963), isolated from a healthy Japanese woman [79].

2.1.3.8.2
Morphological Characteristics

After 7 days at 32ºC on mDixon agar, single colonies (Fig. 2.8a) are 2–3 mm in diameter, flat to slightly wrinkled, dull, pale yellowish-cream, butyrous to somewhat brittle, and with

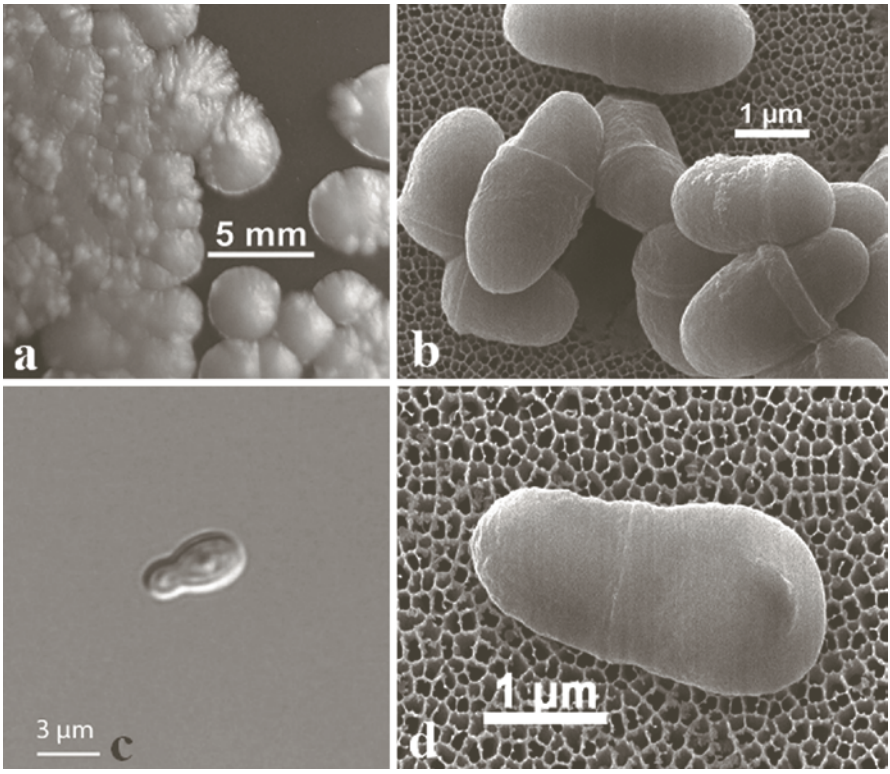


Fig. 2.8 *M. japonica.* (**a**) Flat to slightly wrinkled colonies with a slightly undulate margin; (**b**–**d**) Nomarski's and SEM micrographs of short cylindrical yeasts with a wide and more or less marked budding site

a straight to somewhat undulate margin. Cells are short cylindrical, 3.0–3.5 × 2.0–2.5 µm, with monopolar budding on a broad base (Fig. 2.8b–d). Filaments have not been observed.

2.1.3.8.3
Physiological and Biochemical Characteristics

This species grows at 37°C, but not at 40°C. The catalase reaction is strong, and the species is able to split esculin due to β-glucosidase activity. Only Tweens 60 and 80 are well assimilated, whereas Tween 20 gives a neat ring of colonies at some distance of the well after diffusion of the compound, and Tween 40 is very weakly assimilated at some distance from the well or within the inhibitory area after diffusion of the compound. CrEL is weakly assimilated, but more often only a reactive precipitate appears around the well containing this compound (Plates 2.1-8 and 2.2-8).

2.1.3.8.4
Ecology

Malassezia japonica has been reported from healthy human skin and from the skin of atopic dermatitis patients [79]. The species is not known from another source yet.

2.1.3.9
Malassezia nana Hirai, Kano, Makimura, Yamaguchi & Hasegawa (2004)

2.1.3.9.1
Type Strain

CBS 9557 (JCM 12085), isolated from a cat with otitis externa in Japan [80].

2.1.3.9.2
Morphological Characteristics

After 7 days at 32°C on mDixon agar, single colonies (Fig. 2.9a) are 1.5–2 mm in diameter, cream to yellow, convex, shiny to dull, smooth, butyrous, and have an entire to narrowly folded margin. The cells are ovoid to globose, 3.0-4.0 x 2.0-3.0 µm, with monopolar budding on a relatively narrow base (Fig. 2.9b–d). The species is not known to produce any filaments.

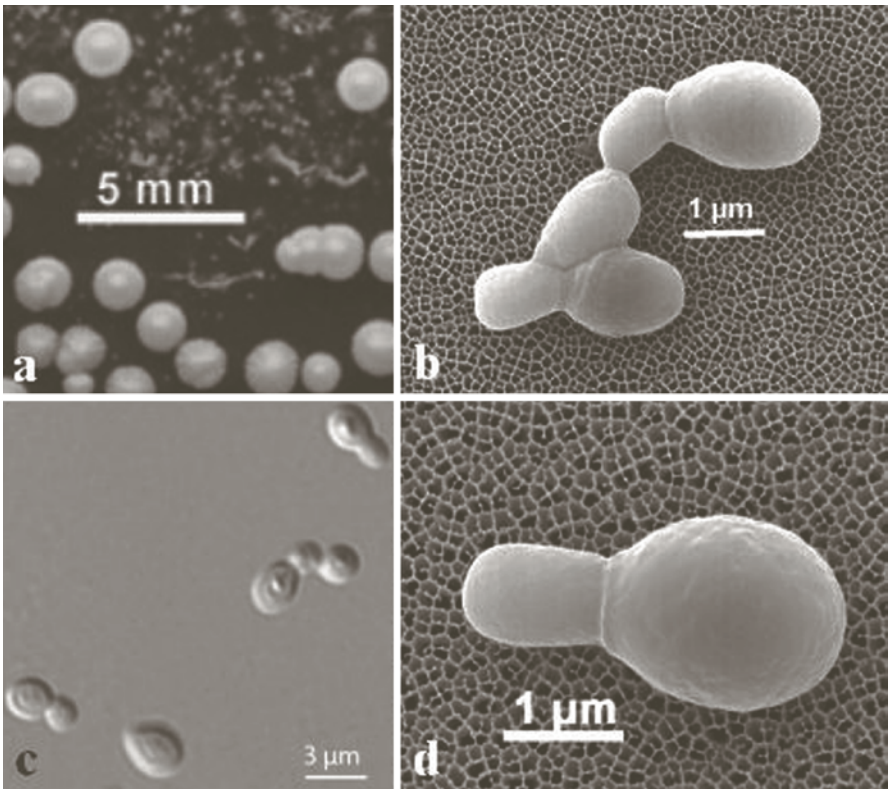


Fig. 2.9 *M. nana.* (**a**) Small, umbonate colonies which are surrounded by a precipitate within the agar; (**b**–**d**) Nomarski's and SEM micrographs of ovoid yeasts with a rather narrow budding site

2.1.3.9.3
Physiological Characteristics

Malassezia nana grows at 37°C, but this is also the maximum temperature for this species, and, hence, growth at 40°C is absent. Catalase and β-glucosidase activities are present. Hirai et al. [80] reported in their description the absence of this latter activity, but we believe that this might have resulted from the technical procedure applied by these authors (see above 2.1.2.3). Tweens 40, 60 and 80 are well assimilated, whereas Tween 20, gives a neat ring of colonies at some distance of the well after diffusion of the compound similarly to *M. equina* and *M. japonica* [81]. CrEL is not utilized (Plates 2.1-9 and 2.2-9). Surprisingly, strains originating from cattle give a complete disk of growth around Tween 20, making the separation with *M. caprae* impossible with the routine methods only. Filaments were not observed.

2.1.3.9.4
Ecology

Malassezia nana is known from healthy cats [77], cats with otitis externa, healthy cows or those with otitis [80].

2.1.3.10
Malassezia equina Cabañes & Boekhout (2007)

2.1.3.10.1
Type Strain

CBS 9969 (MA 146), isolated from healthy anal skin of a horse [18]. This species corresponds to the species that was isolated previously from horse, which was tentatively named *M. equi* by Nell et al. [82], but without providing a valid description, and thus rendering this latter name, invalid.

2.1.3.10.2
Morphological Characteristics

After 7 days at 32°C on mDixon agar, single colonies (Fig. 2.10a) are 2–3 mm in diameter, cream-colored, glistening to dull, butyrous, wrinkled, and with a folded to fringed margin. Crystal precipitates surround the colonies, as was also observed with *M. sympodialis* and *M. dermatis*. Cells are ovoid to ellipsoidal, 3.0–4.5 × 2.2–3.5 µm, with monopolar budding occurring at a narrow base (Fig. 2.10b–d). Filaments have not been observed.

2.1.3.10.3
Physiological and Biochemical Characteristics

Malassezia equina has a maximum temperature at 37°C. The catalase reaction is strong, but in contrast to *M. japonica* and *M. nana,* this species is unable to split esculin. Tweens 40, 60 (note: the white precipitate surrounding these two compounds makes the colony disks look brighter) and 80 are well assimilated, whereas Tween 20, similar to the type strain of *M. nana*, gives a neat ring of colonies at some distance of the well after diffusion of the compound. CrEL is not assimilated, but sometimes a weak precipitate occurs around the well containing this compound (Plates 2.1-10 and 2.2-10). *M. equina*, *M. nana* and *M. japonica* show a neat ring of colonies around Tween 20, but the esculin test discriminates among them, as does the shape of cells, which is ovoid to elongate with a narrow budding site in *M. equina*, short cylindrical with a broad budding site for *M. japonica,* and ovoid and small in size for *M. nana*.

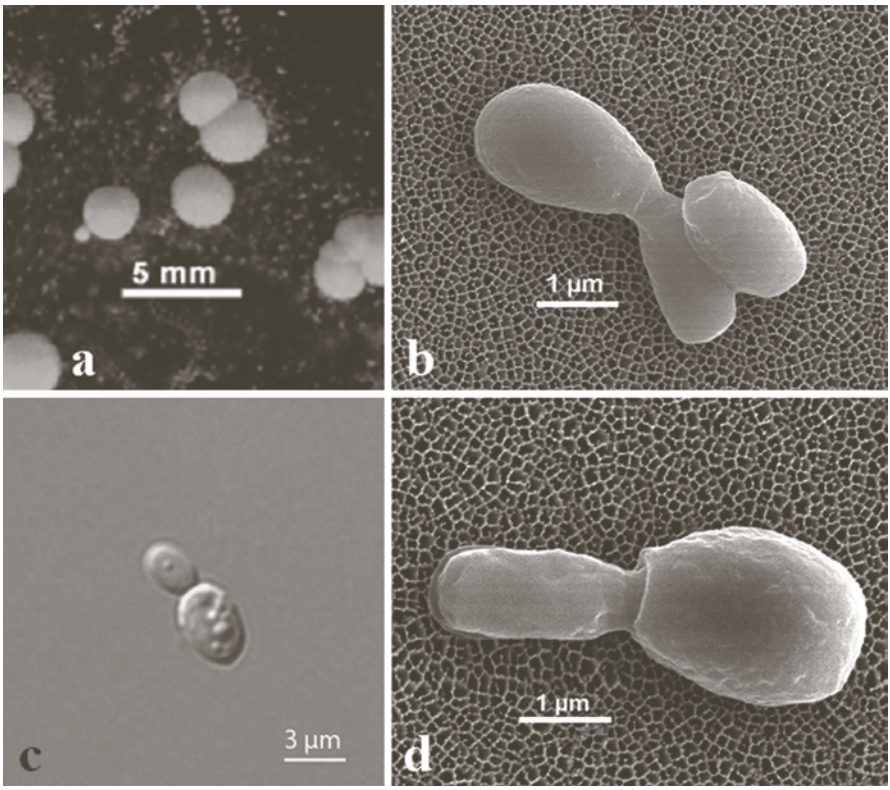


Fig. 2.10 *M. equina.* (**a**) Small, convex colonies that are surrounded by a precipitate within the agar; (**b**–**d**) Nomarski's and SEM micrographs of elongate yeasts with a narrow budding site

2.1.3.10.4
Ecology

So far, *M. equina* is only known from healthy anal skin of horses and from skin of cows in Spain [18].

2.1.3.11
Malassezia obtusa Midgley, Guillot & Guého (1996)

2.1.3.11.1
Type Strain

CBS 7876 (GM215), isolated from human groin [23].

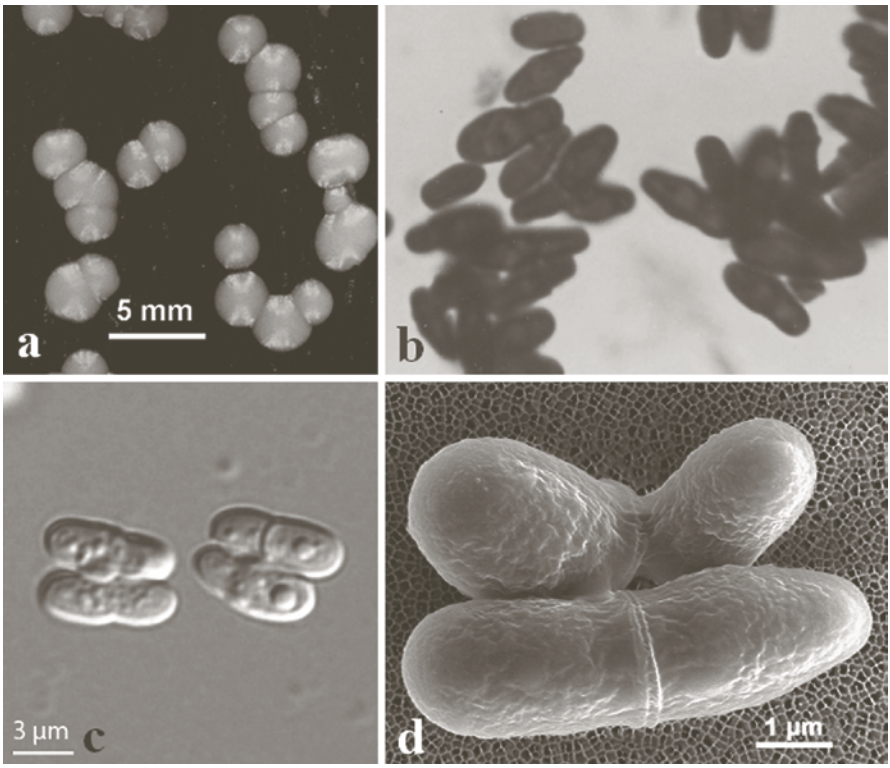


Fig. 2.11 *M. obtusa.* (**a**) Small, convex colonies with an entire to slightly lobate margin; (**b**–**d**) cylindrical to slightly rhomboidal yeasts with a broad budding site (Gram stained in b, Normaski's in c and SEM in d)

2.1.3.11.2
Morphological Characteristics

After 7 days at 32ºC on mDixon agar, single colonies (Fig. 2.11a) are slightly convex, smooth, on average 1.5–2 mm in diameter, shiny or dull, butyrous to pasty, and with the margin, entire to slightly lobate. The cells are cylindrical, somewhat rhomboidal, with obtuse apices, 4.0-6.0 x 1.5-2.0 µm, showing monopolar budding on a broad base (Fig. 2.11b–d). Filaments may be present.

2.1.3.11.3
Physiological and Biochemical Characteristics

Malassezia obtusa is among the most demanding species together with *M. globosa* and *M. restricta.* None of these species grow with individual sources of lipids, although a white precipitate mimics growth around the wells containing Tweens 40 and 60 (Plates 2.1-11 and

2.2-11). Only with fresh isolates and/or very young cultures of *M. obtusa* and *M. globosa*, tiny colonies may appear at some distance from the supplements, particularly due to synergy between two of them (Plate 2.1-11, 12 and Plates 2.2-12). However, the three species can be well identified with other key characteristics. All three species have a maximum temperature at 37°C, but only *M. obtusa* combines positive reactions of catalase and β-glucosidase, whereas *M. globosa* lacks β-glucosidase activity, and *M. restricta* both activities.

2.1.3.11.4
Ecology

Malassezia obtusa is a rare species, which is mainly known from healthy human skin. Together with *M. furfur,* the species has occasionally been isolated from animals, viz., in a case of canine otitis [59] and from healthy horses and goats [60].

2.1.3.12
Malassezia globosa Midgley, Guého & Guillot (1996)

2.1.3.12.1
Type Strain

CBS 7966 (GM35), isolated from PV, UK [23]. *M. globosa* corresponds to the formerly recognized *M. furfur* serovar B [66].

2.1.3.12.2
Morphological Characteristics

After 7 days at 32ºC on mDA, single colonies (Fig. 2.12a) are raised, wrinkled to cerebriform, 3–4 mm in diameter, rough and brittle, pale yellowish, shiny or dull, and with the margin slightly lobate. In primary cultures, colonies are surrounded by an abundant precipitate, as in species of the *M. sympodialis* complex. Cells are spherical, 2.5–8 µm in diameter, and budding is monopolar on a narrow base (Figs. 2.12b–f). In contrast to *M. furfur*, this micromorphology is a stable character in *M. globosa*. Short filaments, reminding germinative tubes of *Candida albicans*, may be present, particularly in primary cultures (Fig. 2.12e, f). On the other hand, pseudo-hyphae are almost always present in PV scales (Fig. 2.12b and see Chap. 6.1). The morphology of *M. globosa* is similar to that of the species known under the old name *Pityrosporum orbiculare* Gordon [83]. Unfortunately, the original type material and isolates designated as *P. orbiculare* on the basis of cell shape and their inability to grow on oleic acid, were not preserved. In the taxonomic revision [23], it was therefore proposed to consider *P. orbiculare* as a doubtful species, which may represent a probable synonym of *M. globosa* and not of *M. furfur*.

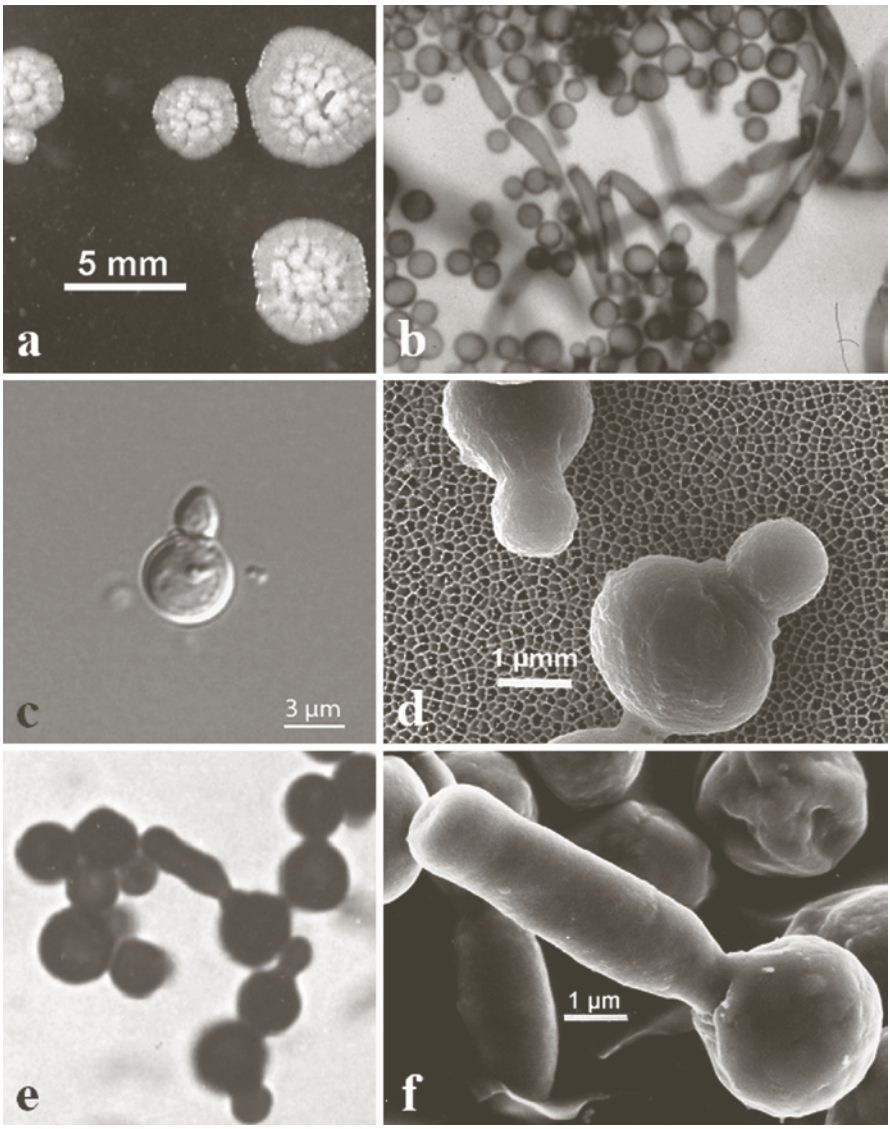


Fig. 2.12 *M. globosa.* (**a**) Large, typically wrinkled to cerebriform colonies with an undulate margin; (**b**) spherical yeasts and filaments in PV scales (i.e., typical spaghetti and meat balls feature) (picture by the courtesy of V. Crespo Erchiga); (**c**–**d**) Normaski's and SEM micrographs showing spherical yeasts with a narrow budding site; (**e**–**f**) Gram stained and SEM micrographs of a primary culture from PV showing typical spherical yeasts, some of them developing a germinative tube

2.1.3.12.3
Physiological and Biochemical Characteristics

Malassezia globosa has a strong catalase activity, similar to that of *M. obtusa*. In contrast to the latter species, *M. globosa* lacks β-glucosidase expression. Growth is limited at 37°C,

and no growth occurs with individual lipid supplements, or may be very weak, with fresh cultures appearing as a ring of tiny colonies at some distance from the well containing Tween 20 (Plates 2.1-12 and 2.2-12) or Tween 80. Due to absence of good growth with individual lipid supplements, and lack of β-glucosidase activity, the species is easily recognized morphologically by its cerebriform colonies and spherical cells.

2.1.3.12.4
Ecology

Malassezia globosa is known from healthy and diseased human skin, mainly from PV [69], but also seborrheic dermatitis and even atopic dermatitis (see Chap. 6.1). One out of four genotypes may be better adapted to such pathologies [84]. The species is also known from animal skin, e.g., cats [72], horses and domestic ruminants [60]. The species has been reported to occur with high frequency in both healthy and diseased bovines with otitis in Brazil [61, 62], with the latter disease known to be associated with nematodes [62]. The species was also isolated from the acoustic meatus of bats [63]. Interestingly, DNA of *M. globosa* has been detected in European soil forest nematodes of the genus *Malenchus* [12], thus suggesting that the occurrence of the species may not be limited to warm blooded animals. Even more surprising was the detection of DNA of the species in soils from Antarctic Dry Valleys [14]. The authors postulated that the occurrence of the yeasts may be associated with nematodes, which are prevalent in Dry Valley soils. The possible interactions between nematodes and *M. globosa* need further study in order to see if nematodes represent a natural reservoir of the species. Further studies, including the characterization of the different rDNA markers and attempts to obtain cultures especially from nematodes, will be necessary to elucidate this possible ecological habitat.

2.1.3.13
Malassezia restricta Guého, Guillot & Midgley (1996)

2.1.3.13.1
Type Strain

CBS 7877 (RA 42.2C), isolated from healthy human skin [23]. *M. restricta* corresponds to the formerly recognized *M. furfur* serovar C [66]. At that time, the authors were not able to demonstrate that they were dealing with different species, but it is interesting to point out that they separated serologically *M. sympodialis* (A), *M. globosa* (B) and *M. restricta* (C), the three species that predominantly occur on human skin, either healthy or lesioned [85].

2.1.3.13.2
Morphological Characteristics

After 7 days at 32ºC on mDA, single colonies (Fig. 2.13a) are small, 1–2 mm in diameter on average, flat to somewhat raised, dull, pale yellowish-brown, hard and brittle, smooth and somewhat ridged near the edge , and with a lobate margin. The cells are ovoid to glo-

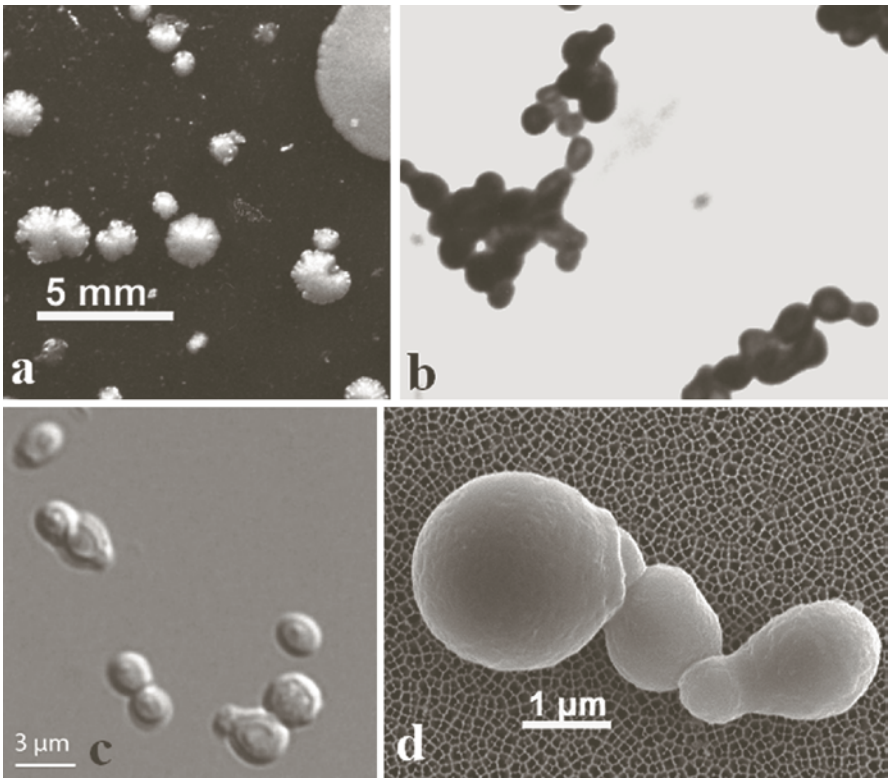


Fig. 2.13 *M. restricta.* (**a**) Very restricted colonies (if compared with *M. sympodialis* (*top right corner*)), that are somewhat raised and have a lobate margin; (**b–d**) Gram stained, Nomarski's and SEM micrographs showing ovoid and globose yeasts with a relatively narrow budding site

bose, 2.5–4 × 1.5–3 µm, with monopolar, percurrent budding on a relatively narrow base (Fig. 2.13b–d). The constant presence of ovoid and globose yeast cells in this species may erroneously suggest a mixture of *M. globosa* and *M. restricta* in the same culture (Fig. 2.13d). However, colonies of both species are sufficiently different to avoid this confusion. Filaments are not known for this species, and, therefore, it may correspond to the species that was described with the old name *P. ovale* [50].

2.1.3.13.3
Physiological and Biochemical Characteristics

Malassezia restricta lacks both, catalase and β-glucosidase activities. This species, which is the most fastidious of the genus, does not grow at 37°C or with any of the lipid supplements. Tweens 40 and 60 can show the presence of a white precipitate appearing like a white disk or a ring around the corresponding wells (Plates 2.1-13 and 2.2-13). Growth with CrEL is always absent.

2.1.3.13.4 Ecology

Malassezia restricta occurs mainly on the head, including scalp, neck, face and ears [85]. Its implication in human disease is not yet elucidated (see Chap. 6) but, as for *M. globosa,* a specific genotype may play a significant role in pathology [86]. Ribosomal DNA sequences (ITS region or D1/D2 LSU domain of the rRNA gene) of the species have been detected from indoor dust in Finland [87], but, surprisingly, also from the gut of beetles in Southern Louisiana, USA [88], forest soil nematodes in Germany [12], and rock beneath a crustose lichen in Norway [13]. These findings suggest that the human body may not be the only habitat of the species. Unfortunately, most of these studies referred to ITS1 only, and attempts to cultivate *M. restricta* were not performed. It is not easy to understand that such a fastidious species can survive without its vital nutritional lipid requirement and below its optimal temperature of growth. The species was also listed to occur in deep sediments in the South China sea [15], but this DNA comparison showed only 85 % ITS sequence identity, which is less than that observed to occur among *Malassezia* species.

2.2 *Malassezia* Phylogeny

Teun Boekhout and Dominik Begerow

Members of the genus *Malassezia*, like many other anamorphic yeasts, are difficult to assign to higher taxonomic levels based on morphological structures. Moore [89] established the name Malasseziales for the basidiomycetous yeasts without ballistospores, in contrast to the Sporobolomycetales that contained genera with actively discharged ballistospores. In the circumscription of the order Malasseziales, Moore included eight genera, namely *Cryptococcus*, *Malassezia*, *Phaffia*, *Rhodotorula*, *Sterigmatomyces*, *Trichosporon*, *Trichosporonoides*, and *Vanrija*. The lack of a unifying character and the negative definition of the Malasseziales already show the difficulties in the grouping of these yeasts in a monophyletic manner.

The availability of ribosomal DNA (rDNA) sequence data allowed a better understanding of the phylogenetic relationship of the yeasts in general. The first more detailed molecular phylogenetic study of heterobasidiomycetous yeasts [90] included *Malassezia* spp. amongst others and clearly separated the genus from species of the Pucciniomycotina (i.e., *Rhodosporidium toruloides*, *Sporodiobolus johnsonii* and *Leucosporidium scottii*) and from members of the Agaricomycotina (i.e., *Filobasidiella neoformans*, *Cystofilobasdium capitatum*, *Phaffia rhodozyma*, *Sterigmatosporium polymorphum*, and some *Trichosporon* spp.). However, due to a limited number of species included, *Malassezia* could not be clearly assigned to an order or class of the Basidiomycota.

During the late 1990s, two groups analyzed the molecular phylogeny of anamorphic basidiomycetous yeasts [91, 92]. Both groups used partial sequences of the large subunit

ribosomal DNA, but while Fell et al. [92] presented an overview of more than 330 yeast strains of basidiomycetes, the second group focused on the anamorph–teleomorph relationship of Ustilaginomycotina. Both studies included members of the Malasseziales, but came up with two different systematic proposals due to different sampling of species. One of the two likely phylogenetic position of Malasseziales based on LSU rDNA sequences is illustrated in Fig. 2.14. The phylogenetic affiliation of Malasseziales within the Ustilaginomycotina (Basidiomycota) is highly supported. However, the relationship between the orders of Ustilaginomycotina is still not resolved in all clades and the support for several groupings is low (Fig. 2.14). This is also reflected in the literature, thus several phylogenetic hypotheses are published. Based on phylogenies of LSU rDNA, several analyses proposed *Malassezia* as part of Exobasidiomycetidae (Exobasidomycetes) like in Fig. 2.14 [91, 93]. Different taxon sampling and other genes could not support this placement or suggested even a grouping with Ustilaginales and Urocystales [94] or sister to all other members of the Ustilaginomycotina. The most recent treatment of fungal taxonomy followed the more cautious proposals and placed the order Malasseziales in the Ustilaginomycotina *incertae sedis* [95].

All 13 species of the genus *Malassezia* form a strongly supported monophyletic group (Figs. 2.14 and 2.15) and support the conclusions based on physiological characters like lipophily and the peculiar cell wall ultrastructure (see Sect. 2.3.1). Based on the analysis of the D1/D2 domains of the large subunit (LSU) rRNA gene, some groups of species are well supported (Figs. 2.14 and 2.15) [also ref. 18]. *M. furfur* forms a monophyletic group with *M. obtusa*, *M. japonica* and *M. yamatoensis*. *M. globosa* is related to *M. restricta*. The group of *M. sympodialis*, *M. nana*, *M. caprae*, *M. dermatis* and *M. equina* form a well supported clade as well, which has been discussed in detail recently [18]. However, *M. slooffiae* and *M. pachydermatis* do not clearly group with any other known species and more detailed studies might present more interesting results. Note that other analyses of partial LSU rRNA gene sequences yielded the same relationships for *M. obtusa* (cited as *M.* species 3) and *M. furfur* [21]. In the ITS analysis, however, the furfur and globosa clades are not separated and form a well supported clade with *M. furfur, M. japonica, M. obtusa, M. pachydermatis, M. yamatoensis* and *M. slooffiae* [18]. Partial sequences of the chitin synthase gene (CHS2) were in agreement with those based on the D1/D2 domains of the LSU rRNA gene and the ITS 1+2 regions [18, 67]. In this analysis, the sympodialis clade comprised *M. sympodialis, M. caprae, M. equina* and *M. dermatis,* whereas *M. nana* formed a basal lineage to this cluster; the furfur clade contained *M. furfur, M. japonica* and *M. obtusa*, and the globosa clade, *M. pachydermatis, M. yamatoensis, M. restricta, M. slooffiae* and *M. globosa*. Partial sequences of the RNA polymerase subunit 1 (RPB1) gene supported the sympodialis clade that also included *M. nana* [18]. All phylogenetic analyses performed so far supported all species recognized, as well as some major clades, but some species, e.g., *M. slooffiae* and *M. pachydermatis*, tend to jump between clades depending on the gene analyzed and the set of other fungi included. This implies that further phylogenetic research is needed to establish the position of these species in the tree of life. A further explanation of these incongruent results may be that hybridization may have occurred during speciation [18].

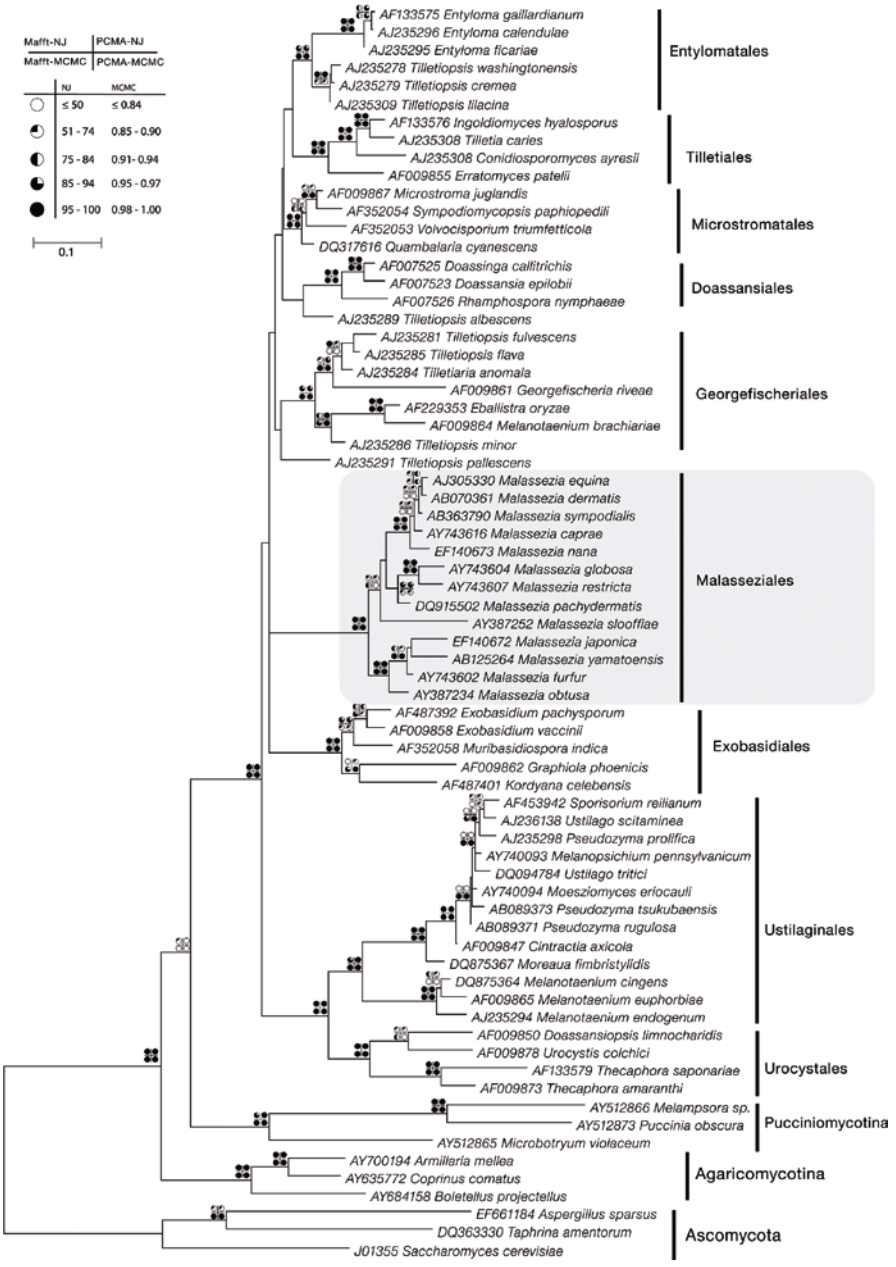


Fig. 2.14 Phylogenetic hypothesis based on partial LSU rDNA sequences of 67 basidiomycetes and three ascomycetes aligned with MAFFT (version 6.525) and analyzed using maximum likelihood in PAUP* 4b10. Support values are calculated based on two different alignment algorithms as implemented in MAFFT and PCMA and two different tree reconstruction methods, viz., neighbor-joining using PAUP* and Markov chain Monte Carlo using MrBayes. Branch lengths represent expected substitutions per base pair

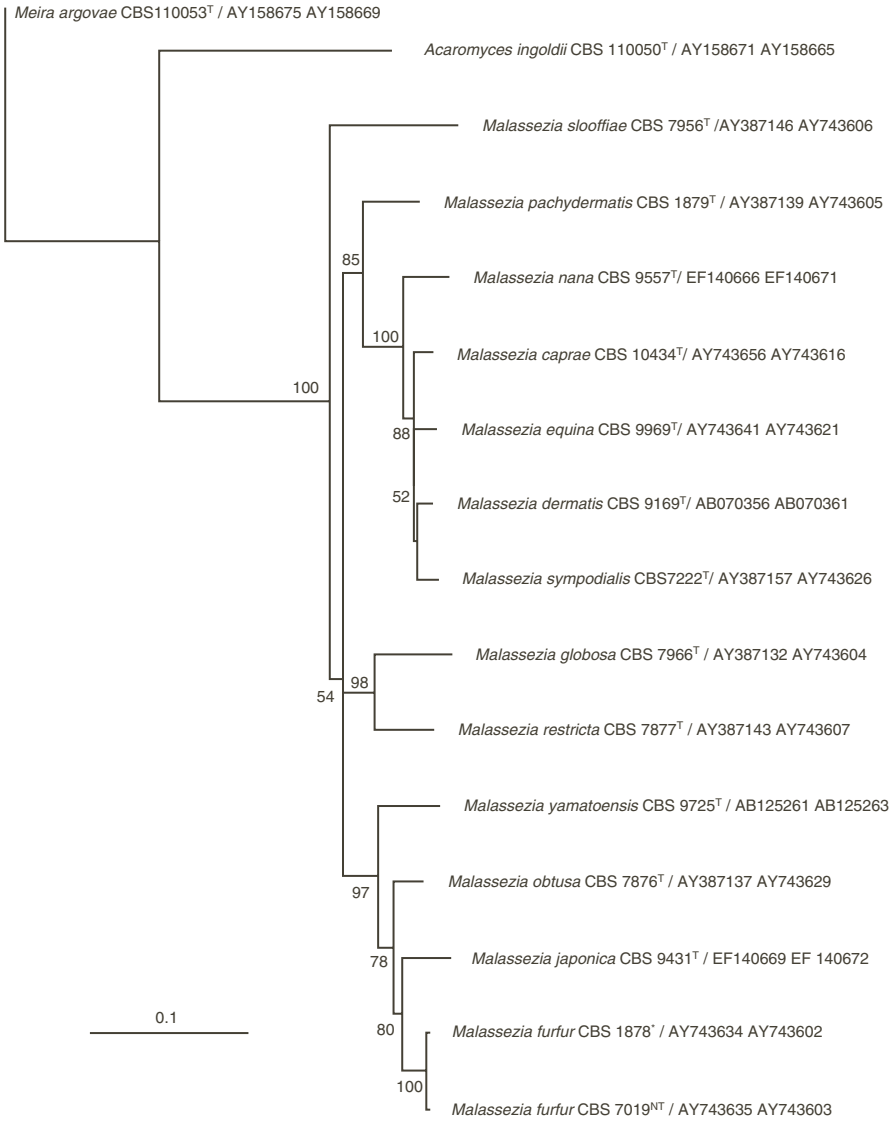


Fig. 2.15 Phylogenetic tree based on concatenated sequences of the ITS1, 5.8s and ITS2 regions of the ribosomal DNA and the D1/D1 part of the LSU rRNA gene generated in Megalign version 7.2.1 (DNAstar Inc.). The tree was generated with PAUP (version 4.0b 10) using the neighbor-joining algorithm with Kimura 2 as a distance measure and 1000 bootstrap replicates. *T* = neotype strain of *M. furfur*; *NT of *Pityrosporum ovale*

2.3 *Malassezia* Ultrastructure

Eveline Guého-Kellermann

The phylogenic studies, as developed above, have demonstrated the affiliation of the genus *Malassezia* to the Ustilaginomycotina of the Basidiomycetes despite the lack of a sexual state (Chap. 2.2). Some other characteristics have been used for a long time to relate these anamorphic yeasts to the Basidiomycetes. The most important of these basidiomycetous markers are the multilamellar cell wall [96, 97] and the monopolar percurrent budding. Unfortunately, due to the absence of hyphae the septal pore structure could not be used to further clarify the taxonomic position of the genus [98].

2.3.1 Cell Wall Ultra-Structure

Transmission electron microscopical (TEM) studies demonstrated that *M. furfur* and *M. pachydermatis* have a thick, electron-dense and multilayered cell wall, which is more or less coated with fibrillar material [99].

This typical basidiomycetous-type cell wall is crossed by a helicoidal translucent band, which arises from regular indentations of the plasma membrane (Fig. 2.16a, b). As far as is known, these cell wall characteristics are unique among the fungi [96–104]. Based on a similar cell wall ultra-structure of the filaments of *M. furfur,* the spherical yeasts (i.e., *Pityrosporum orbiculare*) as observed in PV, and in the oval yeasts (i.e., *Pityrosporum ovale*) as observed in lesions of pityriasis capitis, Keddie [105] suggested that *Pityrosporum* and *Malassezia* might be the same, and, hence, are synonyms. Due to nomenclatural rules, the name *Malassezia* had priority. The freeze-fracture replica technique demonstrated that the plasma-membrane indentations correspond to a regular, helicoidal and left-handed groove [102, 106, 107] which is surrounded by an electron-lucent band, visible as white lines in tangential sections (Fig. 2.16b). This specific parietal ultrastructure is also present in *M. sympodialis* [65], *M. globosa* and *M. restricta* [23] and all other species, as seen by TEM and numerous observations using scanning electron-microscopy (SEM) as well. All *Malassezia* species have a smooth cell wall, but microscopy using a mirror lighting microscope ([108] and Fig. 2.16d) or SEM, of somewhat retracted yeast cells showed, the double helicoidal system appearing on the surface as more or less marked and spaced grooves (see in Chap. 2.1, Figs. 2.1e, 2.4e, 2.5d, 2.6d, and 2.8d of *M. pachydermatis*, *M. sympodialis, M. caprae, M. dermatis* and *M. nana*, respectively). The cell wall of *M. globosa* appears to be somewhat different. By means of the freeze-fracture replication technique, Breathnach et al. [109] showed that the initial major combination of plasma membrane groove and electron-lucent band (Fig. 2.16c) was associated to a minor system of grooves and electron-lucent bands occurring at more

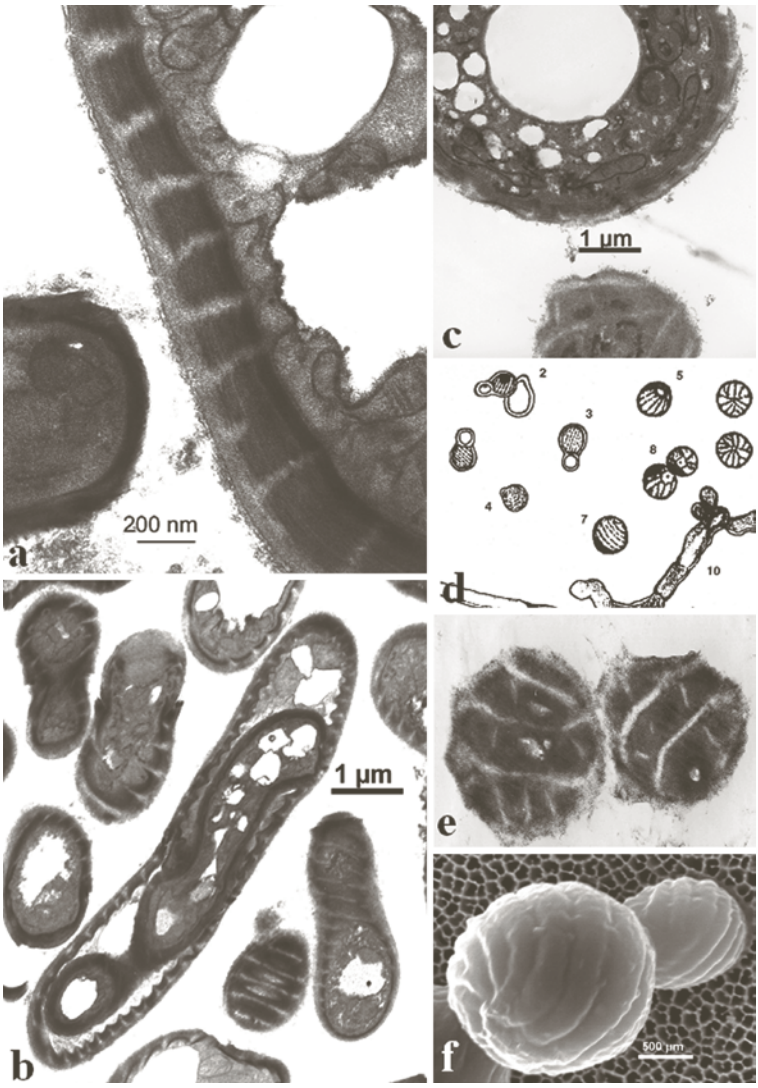


Fig. 2.16 *Malassezia* cell wall ultra-structure. (**a**–**b**) Micrograph of *M. furfur* made by transmission electron-microscopy (TEM). (**a**) The typical multilamellar cell wall with the corrugate invagination of the plasma membrane and its corresponding electron lucent band; (**b**) endospore within elongate yeast cells or short filaments showing the typical lamellate cell wall, and the regularly spaced electron translucent spiral, as can be clearly seen in tangential sections. (**c**–**f**) *M. globosa*. (**c**) Micrograph (TEM) showing the multilamellar cell wall and the electron lucent band; (**d**) drawings after Matakieff [108] of globose yeasts with an helicoidal sculpturing; (**e**) Micrograph (TEM) (courtesy of Hannelore Mittag) of a tangential cut showing the double system of electron lucent bands as described above in Fig. 2.1c; (**f**), Micrograph made by scanning electron-microscopy (SEM) of a retracted yeast and its bud showing the spaced major groove system, that is comparable with Matakieff's drawings

or less right angles. This has also been visualized by TEM analysis of tangential sections of the cell wall (Figs. 2.16c, e). The cell wall thickness appears different from one species to another, but may also be related to the age of the cells and the growth conditions.

2.3.2 Budding Process Ultra-Structure and Endosporulation

The basidiomycetous nature of the genus *Malassezia* is also revealed by its monopolar, blastic and percurrent budding process [100, 101, 104]. Buds emerge from the innermost layer of the wall, and leave a collarette on the mother cell after release (i.e., thus representing a phialidic conidiogenesis) (Fig. 2.17a, b). The scar on the mother cell becomes thicker with the increasing number of collarettes after each subsequent budding (Figs. 2.17c–e, g).

These typical scars of the genus *Malassezia* occur independently of the shape of the yeast cells, but appear more clearly under light microscopy in species having a broad bud site (i.e., *M. pachydermatis, M. furfur, M. yamatoensis, M. slooffiae, M. japonica* and *M. obtusa,* see Chap. 2.1). *M. sympodialis* differs from the other species in that a sympodial budding occurs at the monopolar budding site [97], with the buds appearing alternatively on the left and the right side, thus resulting in a clover leaf-like configuration of the parent cell and its buds (Fig. 2.17f, g). This process, which may also be present in *M. dermatis*, is difficult to visualize under the light microscope and reminds the polyphialides described in hyphomycetes such as *Chloridium* spp. [110].

2.3.3 Other Ultrastructural Characteristics

Endospores (or endoconidia) may be present in *M. furfur* (Fig. 2.16b), and it has been postulated that their presence may suggest an affiliation with teliospore-forming yeasts [103]. Furthermore, it has been suggested that the phenomenon of endosporulation might represent initial steps of basidium development [98, 103]. However, formation of endoconidia has been observed to occur during asexual reproduction in many ascomycetous and basidiomyceous yeast-like fungi. Mittag [111] considered the variable size and shape of the yeast cells within *M. furfur* (CBS 1878 and 6001) due to differences in ploidy. This was indeed confirmed by analysis of chromosomal DNAs using pulsed field gel electrophoresis (for refs. see Chap. 3).

The basidiomycetous affinities of the genus *Malasssezia* are further supported by other cell biological features, such as migration of the nucleus in the bud before mitosis [112]. The distribution of chitin, however, seems restricted to the budding site, similar to that in *Saccharomyces cerevisiae*, whereas it is distributed evenly throughout the cell wall of *Cryptococcus neoformans* [113], another basidiomycetous fungus.

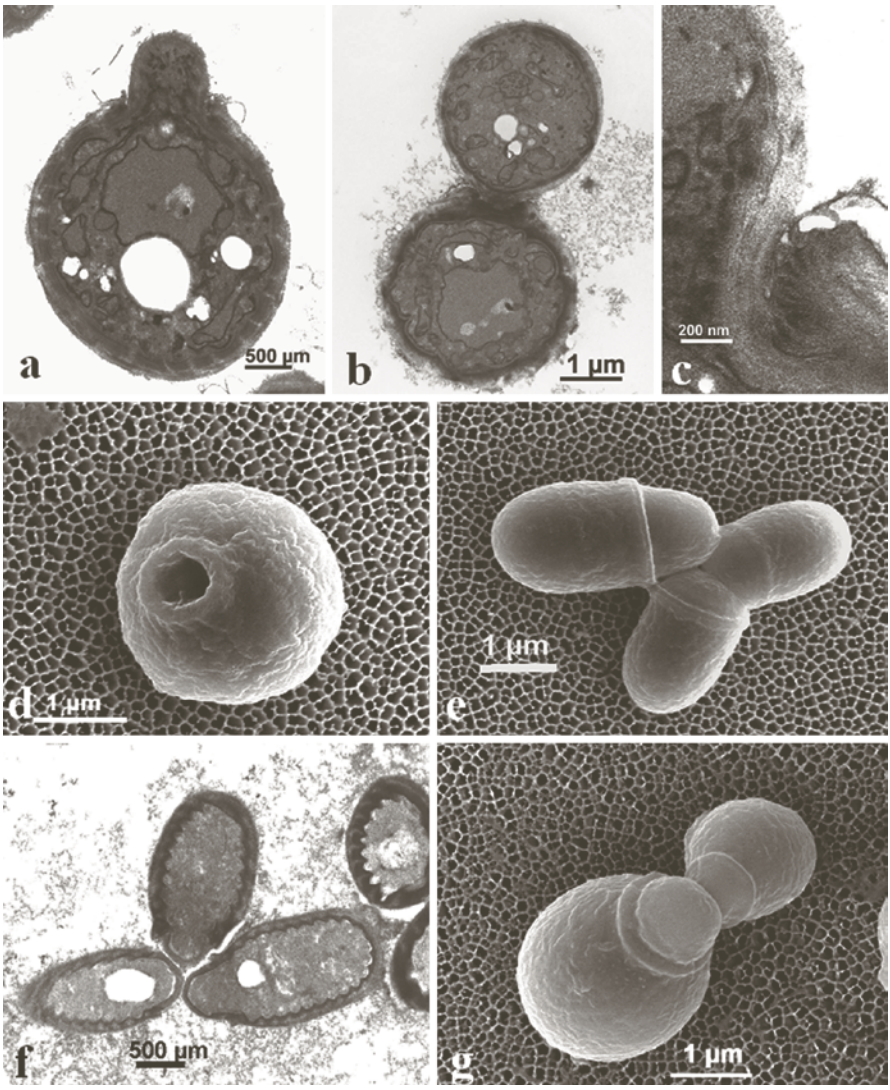


Fig. 2.17 *Malassezia* budding process. (**a**–**d**) *M. globosa*. (**a**–**b**) Micrograph (TEM) showing two states of the monopolar budding process, namely emergence and release of the bud; (**c**) Micrograph (TEM) showing several collarettes resulting from the monopolar percurrent budding; (**d**) Micrograph (SEM) of the budding site with the typical thick budding scar of *Malassezia* spp. (**e**) *Malassezia yamatoensis* (SEM), the youngest yeast cell on the right has a thinner scar. (**f**–**g**) *Malassezia sympodialis*; (**f**) micrographs (TEM) showing the typical clover leaf-like configuration resulting from sympodial budding; (**g**) micrograph (SEM) showing percurrent budding with the youngest bud still emerging

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