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Tween 40-based precipitate production observed on modified chromogenic agar and development of biological identification kit for *Malassezia* species

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We developed a simple identification kit for nine species of *Malassezia* (*M. furfur*, *M. slooffiae*, *M. sympodialis*, *M. restricta*, *M. obtusa*, *M. globosa*, *M. pachydermatis*, *M. dermatis*, and *M. japonica*) based on their biological features. This method utilizes Tween 40-based precipitate production on modified chromogenic agar (CHROMagar) *Malassezia* medium, growth on specific agars (Sabouraud's dextrose agar, Cremophor EL agar, Tween 60-esculin agar), and catalase reactions. This identification kit was verified with 11 type and reference strains of nine *Malassezia* species. An additional 26 clinical isolates were also successfully identified using the kit and the results were confirmed by molecular biological analysis.

Keywords *Malassezia*, identification, chromogenic agar, culture

Introduction

Members of the genus *Malassezia* are among the microbiological flora of the skin of homoiothermic animals. Most species of this genus are lipid-dependent yeasts, which colonize the seborrheic part of skin and are known to be the causative agents of pityriasis versicolor and seborrheic dermatitis.

Malassezia has been re-classified into seven species by molecular biological analysis of nuclear ribosomal DNA/RNA [1,2] and confirmed with mitochondrial ribosomal DNA [3]. A phenotype-based practical method for identification of seven *Malassezia* species was reported by Guillot [4]. Hammer reported production of a precipitate by some *Malassezia* on Dixon's agar [5]: *M. furfur*, *M. obtusa*, and *M. slooffiae* were precipitate-negative, while *M. sympodialis* and *M. globosa* were precipitate-positive. Mayser *et al*. reported esculin hydrolysis and Cremophor EL assimilation of *Malassezia* [6] and these properties have been applied to species differentiation [7].

However, as Sugita *et al*. reported new *Malassezia* species [8,9], it is necessary to develop updated phenotype-based identification methods.

In the present study, we developed a biological feature-based identification kit for nine species of *Malassezia* (*M. furfur*, *M. slooffiae*, *M. sympodialis*, *M. restricta*, *M. obtusa*, *M. globosa*, *M. pachydermatis*, *M. dermatis*, and *M. japonica*) based on combinations of the following: precipitate production on modified chromogenic agar (CHROMagar *Malassezia* agar; CHROMagar, Paris, France) [10], growth on specific agars (Sabouroud's dextrose agar, Cremophor EL agar, Tween 60-esculin agar), and catalase reactions.

Materials and methods

Organisms

Type and standard strains of *Malassezia* (Table 1) were used as references in the present study. An additional 26 fresh isolates of *Malassezia* (Table 2), which were identified by molecular biological analysis, were also used.

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Table 1 Biological characterization based on the kit developed in the present study

Strains	Growth on				Catalase reaction
	CHROM	SDA	TE*	EL	
M. pachydermatis CBS 1879	GP	G	GB	G	+
M. sympodialis CBS 7222	GP	N	GB	N	+
M. globosa CBS 7966	GP	N	N	N	+
M. dermatis JCM11348	GP	N	GN	N	+
M. dermatis JCM11470	GP	N	GN	N	+
M. furfur CBS 1878	G	N	GB	G	+
M. slooffiae CBS 7956	G	N	GN	N	+
M. obtusa CBS 7876	G	N	NB	N	+
M. restricta CBS 7877	G	N	N	N	−
M. japonica M9966	G	N	GB	N	+
M. japonica M9967	G	N	GB	N	+

G, growth; N, no growth; GP, growth and production of precipitate; GB, growth and black zone; GN, growth and no change; NB, no growth and black zone; +, test positive; −, test negative; *, see Figure 2.

Table 2 Comparison of biological identification method and molecular analysis of *Malassezia* species

Strains	Grown on				Catalase	Biological identification	Sequence analysis
	CHROM	SDA	TE	EL			
Asc1	GP	G	GB	G	+	*M. pachydermatis*	*M. pachydermatis*
Asc8	GP	G	GB	G	+	*M. pachydermatis*	*M. pachydermatis*
Asc20	GP	G	GB	G	+	*M. pachydermatis*	*M. pachydermatis*
Asc21	GP	G	GB	G	+	*M. pachydermatis*	*M. pachydermatis*
9978	GP	N	GB	N	+	*M. sympodialis*	*M. sympodialis*
9979	GP	N	GB	N	+	*M. sympodialis*	*M. sympodialis*
No.15	GP	N	N	N	+	*M. globosa*	*M. globosa*
9970	G	N	GB	G	+	*M. furfur*	*M. furfur*
9971	G	N	GB	G	+	*M. furfur*	*M. furfur*
Sp3	G	N	GB	G	+	*M. furfur*	*M. furfur*
Sp4	G	N	GB	G	+	*M. furfur*	*M. furfur*
Sp5	G	N	GB	G	+	*M. furfur*	*M. furfur*
Sp6	G	N	GB	G	+	*M. furfur*	*M. furfur*
Sp8	G	N	GB	G	+	*M. furfur*	*M. furfur*
Sp9	G	N	GB	G	+	*M. furfur*	*M. furfur*
Sp12	G	N	GB	G	+	*M. furfur*	*M. furfur*
Sp13	G	N	GB	G	+	*M. furfur*	*M. furfur*
Sp14	G	N	GB	G	+	*M. furfur*	*M. furfur*
Sp15	G	N	GB	G	+	*M. furfur*	*M. furfur*
9980	G	N	GN	N	+	*M. slooffiae*	*M. slooffiae*
9981	G	N	GN	N	+	*M. slooffiae*	*M. slooffiae*
9974	G	N	NB	N	+	*M. obtusa*	*M. obtusa*
9975	G	N	NB	N	+	*M. obtusa*	*M. obtusa*
Sp1	G	N	NB	N	+	*M. obtusa*	*M. obtusa*
Sp2	G	N	N	N	−	*M. restricta*	*M. restricta*
Sp11	G	N	N	N	−	*M. restricta*	*M. restricta*

G, growth; N, no growth; GP, growth and production of precipitate; GB, growth and black zone; GN, growth and no change; NB, no growth and black zone; +, test positive; −, test negative.

Culture media

Strains of *Malassezia* were maintained on modified Leeming and Notman Agar (LNA), composed (per liter) of 10 g of peptone (Oxoid, Basingstoke, UK), 10 g of glucose, 2 g of yeast extract (Oxoid), 8 g of ox bile (Oxoid), 10 ml of glycerol, 0.5 g of glycerol monostearate, 5 ml of Tween 60, 20 ml of olive oil, and 15 g of agar (Oxoid), and sterilized by autoclaving.

The following specific agars were used in the present study. Modified CHROMagar *Malassezia* agar was composed (per liter) of 56.3 g of CHROMagar *Malassezia* basal medium (CHROMagar) [10] and 10 ml of Tween 40; Sabouroud's dextrose agar (SDA) was composed (per liter) of 10 g of mycological peptone, 40 g of glucose, and 15 g of agar; Cremophor EL agar (EL slant) was composed (per liter) of 65 g of SDA and 10 ml of Cremophor EL (Sigma); Tween 60-esculin agar (TE slant) was composed (per liter) of 10 g of peptone, 10 g of glucose, 2 g of yeast extract, 5 ml of Tween 60, 0.5 g of ferric ammonium citrate, 1 g of esculin, and 15 g of agar.

Identification methods

All test strains of *Malassezia* species were inoculated on modified CHROMagar *Malassezia* agar and specific agars (SDA, EL slant, TE slant) and then incubated at 32°C for 4 days before observation. Fresh cultures grown on modified CHROMagar *Malassezia* were subjected to catalase test with 3% hydrogen peroxide.

Molecular analysis

DNA was extracted by the procedure of Makimura *et al*. [11]. The internal transcribed spacer 1 (ITS1) region was sequenced directly from PCR products using the primer pair, 18SF1 and 58SR1 [12]. The PCR products were sequenced with an ABI PRISM 310 Genetic Analyzer according to the manufacturer's instructions.

Results

Tween 40-based precipitate production on modified CHROMagar Malassezia

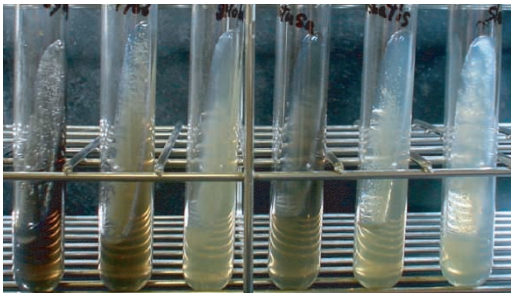
All of the type and reference strains of *M. pachydermatis*, *M. sympodialis*, *M. globosa*, and *M. dermatis* produced precipitates on incubation at 32°C for 4 days on modified CHROMagar *Malassezia* (CHROM; Fig. 1–1, 1–2, 1–3, and 1–8). Other type and reference strains (*M. furfur*, *M. slooffiae*, *M. obtuse*, *M. restricta*, and *M. japonica*) did not produce such precipitates (Fig. 1–4, 1–5, 1–6, 1–7, and 1–9).

Fig. 1 Precipitate production on modified CHROMagar *Malassezia*. Nine *Malassezia* species were incubated for 4 days at 32°C on CHROMagar *Malassezia*. Numbers indicate each species, as shown below: (1) *M. pachydermatis*; (2) *M. sympodialis*; (3) *M. globosa*; (4) *M. furfur*; (5) *M. slooffiae*; (6) *M. obtusa*; (7) *M. restricta*; (8) *M. dermatis*; (9) *M. japonica*. Note the precipitate production by colonies of No. 1, 2, 3, and 8.

Differentiation using specific agars for type and reference strains

The results of application of the kit to the eleven type and reference strains of nine species are shown in Table 1.

1. Strains of the precipitate-producing group on CHROM: After incubation, *M. sympodialis* and *M. pachydermatis* produced a black zone around the colonies due to esculin hydrolysis products and ferrous iron in TE slants (Fig. 2–1). However, *M. dermatis* did not produce such a zone (Fig. 2–5). *M. globosa* did not grow on TE slants (Fig. 2–3). Only *M. pachydermatis* grew on SDA. *M. restricta* was the only catalase-negative species.
2. Strains of the non-precipitate producing group on CHROM: All of the precipitate-negative strains were positive for catalase reaction. After incubation, *M. japonica* showed production of a black zone around the colonies on TE slants (Fig. 2–2). On the other hand, *M. slooffiae* did not produce such a zone (Fig. 2–6). *M. obtusa* did not grow but



No.	1	2	3	4	5	6
Growth	+	+	−	−	+	+
Black zone	+	+	−	+	−	−

Fig. 2 Growth and black zone production in TE slant. *Malassezia* species was incubated on TE slant at 32°C for 4 days. Nos. (1) *M. sympodialis* CBS7222; (2) *M. japonica* M9966; (3) *M. globosa* CBS7966; (4) *M. obtusa* CBS7876; (5) *M. dermatis* JCM11348; (6) *M. slooffiae* CBS7956; +, growth or black zone positive; −, growth or black zone negative.

produced a black zone on TE slants (Fig. 2–4). Only *M. furfur* grew on EL slants.

Identification of clinical isolates

Twenty-six fresh isolates were identified according to their pattern of biological properties as shown in Table 1. All patterns of biological properties of isolates are shown in Table 2. Four strains of *M. pachydermatis*, 2 of *M. sympodialis*, 1 of *M. globosa*, 12 of *M. furfur*, 2 of *M. slooffiae*, 3 of *M. obtusa*, and 2 of *M. restricta* grew successfully and were identified using the kit. All identifications were confirmed by molecular biological analysis.

Discussion

A culture-based identification kit for *Malassezia* species was developed in the present study. In the kit, modified CHROMagar *Malassezia* was used as the primary culture medium. CHROMagar *Malassezia* basal medium is based on CHROMagar *Candida* modified to support the growth of *Malassezia* species, as reported previously by our group [as LN-CHROM; 10] and licensed to CHROMagar Inc. We found that addition of Tween 40 to CHROMagar *Malassezia* basal medium caused formation of a precipitate in the agar for some specific *Malassezia* species, similar to the observations reported previously on Dixon's agar by Hammer and Riley [5]. This modification of CHROMagar *Malassezia* was also licensed to the above company. In addition to their report, it was shown that the new species, *M. dermatis*, reported by Sugita *et al*. [8], also produced a precipitate. Although, *M. dermatis* and *M. japonica* are new species reported by Sugita *et al*. [8,9], their biological properties resembled those of *M. slooffiae* and *M. sympodialis*, respectively, but their characteristics with regard to precipitation on modified CHROMagar *Malassezia* were different.

Using modified CHROMagar *Malassezia* with three specific agars and catalase reactions, 11 type and reference strains and 26 clinical isolates were successfully identified easily, quickly, and at reasonable cost without any expensive or special equipment. Recently, *M. nana* and *M. yamatoensis* were reported as new species of *Malassezia* [13,14]. However, these species are thought to be rather rare, and we are currently planning to develop a means of identify these species in a future study. It is also underway in our laboratory that the evaluation of identification using this kit for fresh isolates of *M. dermatis* and *M. japonica* as same as the newest two species. The results presented here indicate that modified CHROMagar *Malassezia* and this kit will be powerful routine tools for the identification of *Malassezia* species.

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