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NOTE

Taxonomy of *Eurotium* Species Isolated from Meju

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Eurotium **strains were isolated from 77 loaves of meju (dried fermented soybeans), in various regions of Korea from 2008 to 2010. Morphological characteristics and DNA sequences of β-tubulin were examined. They were identified as *Eurotium amstelodami*, *E. chevalieri*, *E. herbariorum*, *E. repens*, *E. rubrum*, and *E. tonophilum*. Of these species, *E. chevalieri* and *E. tonophilum* had not been previously reported in association with meju. *E. chevalieri* and *E. repens* were the species isolated most frequently. This paper summarizes the morphological characteristics of six *Eurotium* species and provides key to identify the species from meju.**

Keywords: *Eurotium*, Meju, identification, *E. chevalieri*, *E. tonophilum*

Soybeans and soybean products are a major source of protein in Southeast Asian countries. In Korea, the most important fermented soybean products are soybean paste (doenjang) and soy sauce (ganjang) (Lee, 1995) which are derived from meju, a brick of dried fermented soybeans. These foodstuffs have been manufactured at home using traditional methods for thousands of years in Korea, and have recently found their way into the spotlight due to the tide of Hansik (Korean food) globalization. Fungi in meju play an important role degrading macromolecules into smaller nutrient molecules. Although many papers regarding the fungal flora of meju have been reported since the 1950s (Hahn and Park, 1957; Hahn and Kim, 1962; Yihn and Lee, 1968; Cho and Lee, 1970; Sakurai *et al.*, 1985; Lee, 1995), the fungi involved were not precisely identified into species level and the identification was based only on morphological characteristics. Furthermore, the fungal strains unfortunately were not preserved in any Korean collections or institutes for use as genetic resources. As such, we attempted to collect fungal resources from meju in an effort to elucidate the fungal flora. During the course of this study, *Eurotium* strains were isolated with a high frequency and from a large proportion of the meju tested. *Eurotium* species are xerophilic, show high proteolytic activity on meat, and are generally regarded as benign fungi, free of mycotoxin (Pitt and Hocking, 2009). Some *Eurotium* species have been used in food production, for example as starter culture for Katsuobushi and fish sauce (Pitt and Hocking, 2009). While *Eurotium* has potential as an effective starter for fermented foods and is frequently found in meju, it has only been described in two papers to date in relation to meju. Sakurai *et al*. (1985) reported the species, *Eurotium amstelodami*, *E. herbariorum*, and *E. repens* from meju, but did not provide a description of associated characteristics. Yun *et al*. (2009) identified and characterized only *Eurotium rubrum* from improperly fermented meju. The objectives of this study were: 1) to collect and identify *Eurotium* species from meju from diverse regions of Korea; 2) to summarize taxonomic characteristics of *Eurotium* species of meju and provide a method to identify the species; and 3) to provide researchers with *Eurotium* strains as genetic resources for developing good starters for meju.

We collected 77 loaves of meju from diverse regions of Korea, and isolated *Eurotium* species from them. For the isolation of *Eurotium* strains, two methods were used. One process involved direct examination of fungi on meju and direct plating of particles of meju or pieces of fungal material on Malt Extract Agar with 20% Sucrose (ME20S) [50 g Malt Extract Agar (Oxoid CM0059), 200 g sucrose, 1 L DW] or Dichloran 18% Glycerol Agar (DG18) [31.5 g Dichloran-Glycerol Agar Base (Oxoid CM0729), 220 g glycerol, 0.1 g chloramphenicol, 1 L DW] (Samson *et al.*, 2004). The other method involved dilution plating on Dichloran Rose Bengal Chloramphenicol Agar (DRBC) [32 g Rose-Bengal Chloramphenicol Agar Base (Oxoid CM0549), 0.002 g Dichloran, 0.1 g chloramphenicol, 1 L DW] and DG18 (Samson *et al*., 2004). A total of 112 strains were isolated from 77 loaves of meju. Table 1 shows representative *Eurotium* strains of them, and the strains were deposited into Korean Agricultural Culture Collection (KACC) in National Academy of Agricultural Science.

For the identification of *Eurotium* species, morphological characteristics including scanning electron microscopy (SEM) of ascospore and molecular characteristics of DNA sequences of β-tubulin and rDNA ITS were examined. For macro-morphological observations, several agar media were used: DG18 (both on 25°C and 37°C), MEA (50 g Oxoid CM0059) (25°C), ME20S (25°C), and Yeast Extract Sucrose Agar (YES) (20 g yeast extract, 150 g sucrose, 20 g agar, 1 L DW) (25°C). For

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Table 1. *Eurotium* strains isolated from 77 loaves of meju

Eurotium species	No. of strains	No. of meju loaves	Provinces[a] collected from	Representative strains	
				Isolate no.	β-Tubulin accession no.
E. amstelodami	14	11	Gg, Gw, Gb, Jb	KACC 45337	HQ245688
				KACC 45338	HQ245690
				KACC 45339	HQ245691
				KACC 45340	HQ245693
				M 1040	HQ245692
				M 1126	HQ245689
E. chevalieri	39	21	Gg, Cb, Cn, Gb	KACC 45342	HQ245703
				KACC 45343	HQ245707
				KACC 45344	HQ245708
				KACC 45346	HQ245709
				KACC 45347	HQ245706
				M 1071	HQ245705
				M 1090	HQ245704
				M 1091	HQ245702
E. herbariorum	12	9	Gg, Cb, Gb	KACC 45348	HQ245681
				KACC 45349	HQ245695
E. repens	33	22	Gg, Gb, Jb	KACC 45351	HQ245701
				KACC 45352	HQ245698
				KACC 45353	HQ245694
				KACC 45354	HQ245696
				KACC 45355	HQ245700
				KACC 45358	HQ245697
				KACC 45359	HQ245699
E. rubrum	12	8	Gg, Gb, Jb	KACC 45360	HQ245682
				KACC 45362	HQ245683
				KACC 45363	HQ245685
				M 1033	HQ245686
				M 1067	HQ245687
				M 1099	HQ245684
E. tonophilum	2	1	Gb	KACC 45365	HQ245680

[a] Abbreviations of provinces: Gg, Gyeonggi; Gw, Gangwon; Cb, Chungbuk; Cn, Chungnam; Gb, Gyeongbuk; Gn, Gyeongnam; Jb, Jeonbuk; Jn, Jeonnam

micro-morphological observations, microscopic mounts were made from DG18 colonies. For SEM observations, mature cleistothecia, incubated >2 weeks were used. For molecular characterization, the DNA sequences of β-tubulin (primers bt2a and bt2b; Glass and Donaldson, 1995) and rDNA ITS (primers ITS5 and ITS4; White *et al*., 1990) were used. In order to determine the taxonomic positions of the meju strains, the DNA sequence of β-tubulin and rDNA ITS of Peterson (2008) were obtained from GenBank and compared. DNA data were analyzed using the Tamura-Nei parameter distance calculation model, which was then used to construct the Neighbor-Joining (NJ) tree with MEGA version 4.1 (Tamura *et al.*, 2007). To determine the support for each clade, bootstrap analysis was performed with 1,000 replications. The β-tubulin phylogenetic tree of the *Eurotium* species is shown in Fig. 1.

KACC 45337-45340, M1040 and M1126 were clustered with *E. amstelodami*^{T} NRRL 90, *Aspergillus hollandicus* NRRL 25850, *E. heterocaryoticum*^{T} NRRL A-13891, and *E. montevidense*^{T} NRRL 108. *A. hollandicus* is the anamorphic name of *E. amstelodami* (index fungorum), and both *E. heterocaryoticum* and *E. montevidens*e are synonym of *E. amstelodami* (Peterson, 2008). The six strains appeared as dull green colonies due to green conidia, but partially yellow with cleistothecia on DG18 agar (Fig. 2Ab). Their ascospores were lenticular, 4.0-5.0 μm, and had conspicuous short ridges, clear wide furrow and convex with rough surface (Fig. 2Ac). Based on these molecular and morphological characteristics, they were identified as *Eurotium amstelodami* L. Mangin. The species was observed with dark green conidiation or yellow cleistothecia on the surface or shallow inside of meju (Fig. 2Aa).

KACC 45342-4, KACC 45346-7, M1071, M1090, and M1091 were clustered with *E. chevalieri*^{T} NRRL 78 (Fig. 1). The eight strains from meju showed yellow green colonies owe to green conidia, yellow cleistothecia and hyphae on DG18 agar (Fig. 2Bb). Growth at 37℃ was faster than at 25℃ on DG18 after 7 day incubation. Their ascospores were lenticular, 4.0-5.0 μm, and had thin and flexuous ridges, wide furrows and convex with smooth surfaces (Fig. 2Bc). Based upon these characteristics, they were identified as *Eurotium chevalieri* L. Mangin. The species was found with dark green conidiation on the surface or shallow inside of meju (Fig. 2Ba).

In the case of KACC 45348 and 45349, colonies on DG18 agar were highly floccose with aerial hyphae (Fig. 2Cb). They

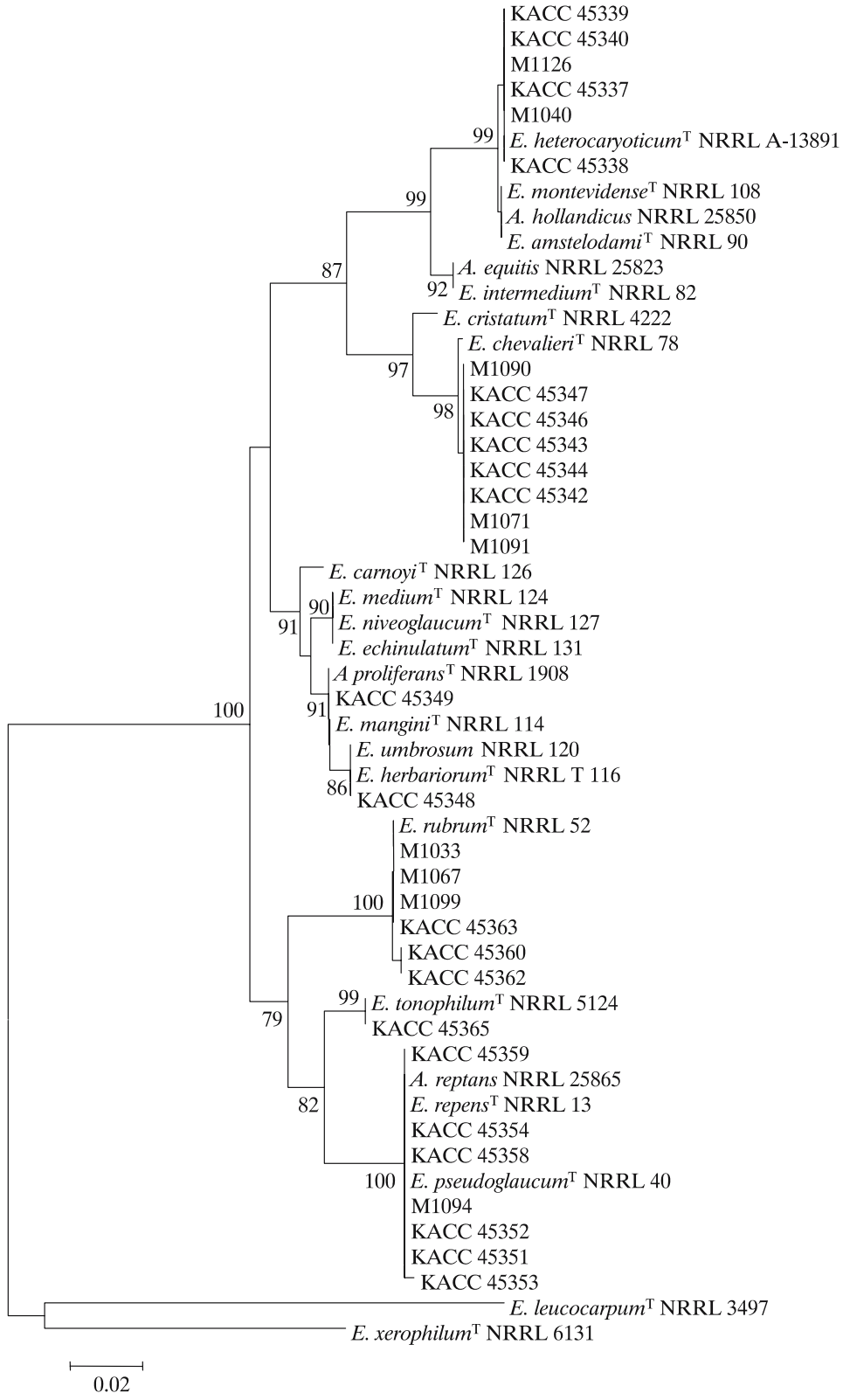

Fig. 1. Neighbor-joining tree depicting taxonomic position of *Eurotium* strains from meju. The tree is based on DNA sequences of partial β-tubulin gene segments. Numbers at the nodes are bootstrap values and the subscript T denotes type strain of the species. Reference sequences are from Peterson (2008).

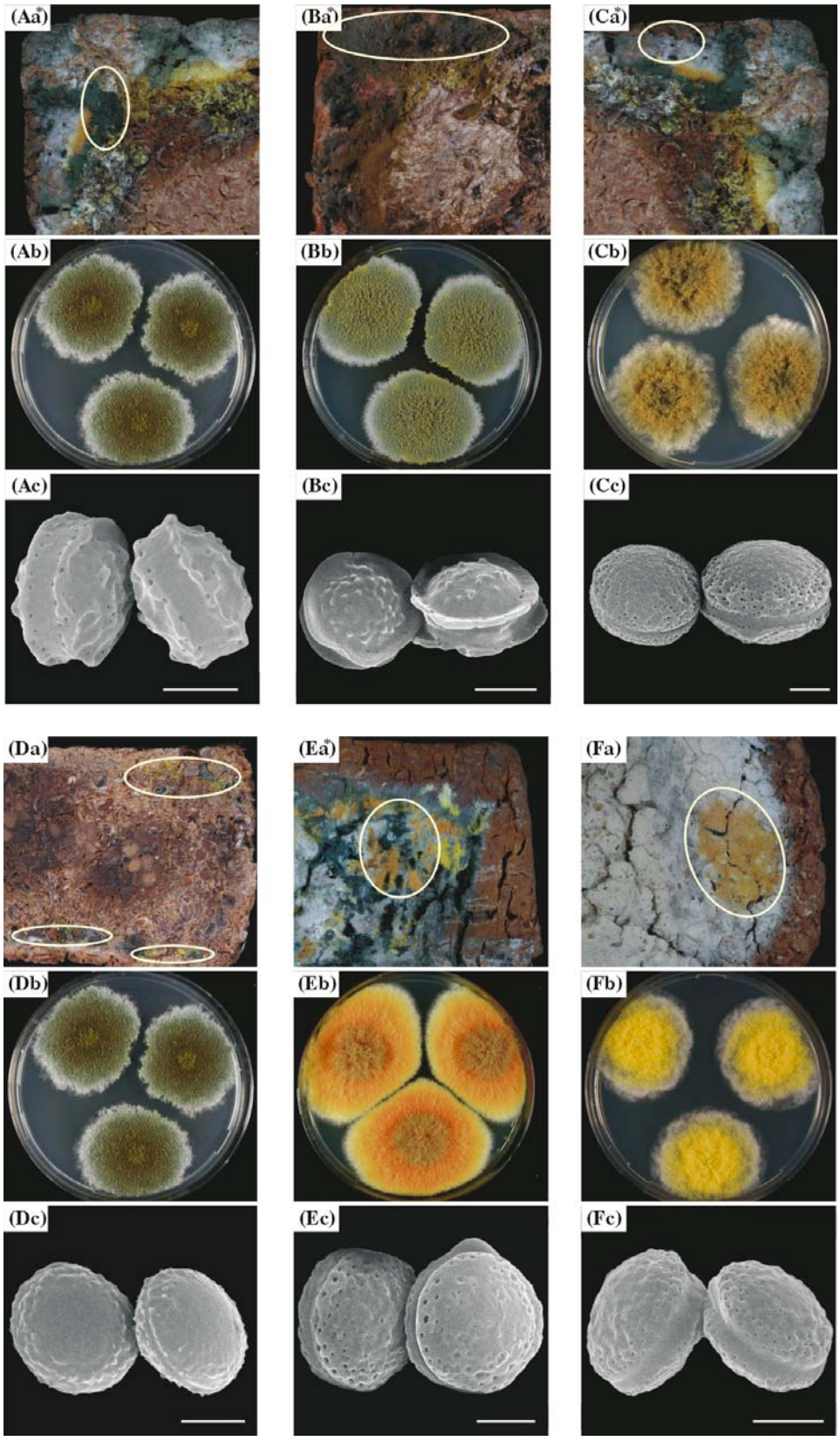

Fig. 2. Morphological characteristics of *Eurotium* species from meju. Symptoms on meju (a), colony on DG18 (b), and ascospores (c) of *Eurotium* species. A, *Eurotium amstelodami*; B, *Eur. chevalieri*; C, *Eur. herbariorum*; D, *Eur. repens*; E, *Eur. rubrum*; F, *Eur. tonophilum*. Bar, 2 μm. The meju pictures (Aa, Ba, Ca, Ea) were taken after 2 weeks storage on room temperature from collection.

did not grow on DG18 at 37°C and germinated but failed to grow beyond the point of inoculation on MEA at 25°C. Their ascospores were lenticular, (5.5) 6.0-7.0 μm, and had no clear crest, clear wide furrows and convex with smooth surfaces (Fig. 2Ac). The two strains were clustered with *Aspergillus proliferans*^{T} NRRL 1908, *E. mangini*^{T} NRRL 114, *E. umbrosum* NRRL 120, and *E. herbariorum*^{T} NRRL 116. *A. proliferans* is a strict anamorphic species (Raper and Fennell, 1965), *E. manginii* is a synonym of *E. herbariorum* (Peterson, 2008), and *E. umbrosum* has large ascospores (7.5-8.5 μm). Based upon these results, they were identified as *Eurotium herbariorum* (F.H. Wigg.) Link. The species was observed with yellow cleistothecia on the shallow inside of meju (Fig. 2Cc).

KACC 45351-KACC 45355, KACC 45358, and KACC 45359 were clustered with *E. repens*^{T} NRRL 13, *A. reptans* NRRL 25865, and *E. pseudoglaucum*^{T} NRRL 40 (Fig. 1). *E. pseudoglaucum* is synonym of *E. repens* (Peterson, 2008), and *A. reptans* is the anamorph of *E. repens* (index fungorum). These isolates showed dark green to yellow or orange color, owing to green conidial heads, and yellow to orange cleistothecia and hyphae on DG18 agar (Fig. 1Db). They did not grow at 37°C on DG18. Their ascospores were lenticular, 4.5-5.5 μm, and had no equatorial ridge, and had narrow furrow which can be seen only trace (Fig. 2Dc). Based upon these characteristics, they were identified as *Eurotium repens* de Bary. The species was observed with dark green conidiation on the surface and shallow inside of meju, and also found with yellow cleistothecia inside meju (Fig. 2Da).

KACC 45360, KACC 45362-3, M1033, M1067, and M1099 were clustered with *E. ruburum*^{T} NRRL 52. The colonies on DG18 agar were dull green, yellow, orange, or red. The orange and red colors were a result of the hyphae. They did not grow at 37°C. Their ascospores were large lenticular (5.0-6.0 μm), and had no ridge, but clear wide furrows (Fig. 2Ec). Based upon these characteristics, they were identified as *Eurotium ruburum* W. Bremer. The species was found with orange hyphae on surface or white hyphae on shallow inside of meju (Fig. 2Ea).

KACC 45365 was clustered with *E. tonophilum*^{T} NRRL 5124 (Fig. 1). The colony of the isolate was yellow owe to abundant cleistothecia and their surrounding hyphae on DG18. It did not grow at 37°C on DG18. The ascospores were lenticular (5.0-5.5 μm), and had wide definite furrows (Fig. 2Fc). From these characteristics, it was identified as *Eurotium tonophilum* Ohtsuki. The species was found with yellow cleistothecia and hyphae on the surface of meju (Fig. 2Fa).

Of the six species, *E. chevalieri* (39 strains from 29 loaves of meju) and *E. repens* (33/22) were isolated more frequently than the other species from meju (Table 1), and *E. chevalieri* and *E. tonophilum* had not been previously reported in association with meju. Major morphological characteristics of the *Eurotium* species from meju are summarized in Table 2. For easy identification of *Eurotium* species from meju by morphological characters, a dichotomous key was produced as follows:

Dichotomous key to identify Eurotium species from meju

1. Mycelium is only white. Colony color is green and yellow because of conidia and cleistothecia, respectively. Ascospores

Table 2. Major morphological characteristics of *Eurotium* species from meju

Eurotium	Growth on Media (mm)					Stipe		Vesicle		Conidia			Cleisto-		Ascospore			
	DG18 (37)	DG18	MEA	ME 20S	YES	Length (μm)	Surface[a]	Diameter (μm)	Shape[b]	Length (μm)	Shape[b]	Texture[c]	thecia size (μm)	Asci (μm)	Size (μm)	Ridge	Furrow	Texture[c]
amstelodami	22-32	37-44	11-19	22-39	32-40	120-300	Sm	15-30	gl-spa	4-6	sbg-br el	rf	100-160	12-15	4.0-5.0	clear, stiff, low	wide, clear	rf
chevalieri	>55	32-51	17-23	43-70	46-53	300-540	Sm	25-40	gl-py	5-7	sbg, oval	rf	60-150	10-15	4.0-5.0	clear, flexible, high	wide, clear	sm
herbariorum	X	39-48	<6	27-38	23-25	<300	Sm	12-45	gl-py	4-7	gl, sbg, br-el	rf	100-200	10-12	5.5-7.0	no	wide, clear	sm
repens	X	45-60	17-23	46-61	33-46	200-500	Sm	15-38	gl-py	5-7	sbg, oval	rf	60-200	10-12	4.5-5.5	no	narrow, only trace	sm
rubrum	X	38-70	12-24	50-65	33-47	300-600	Sm	12-23	gl-py	5-8	sbg, oval	rf	60-100	10-12	5.0-6.0	no	wide, clear	sm
tonophilum	X	46	X	38	29	200-300	Sm	18-35	gl-spa	5-7	glo, sbg	rf	50-200	11-13	5.0-5.5	no	wide, clear	sm

[a] Surface: sm, smooth.
[b] Shape: gl, globose; spa, spathulate; py, pyriform; sbg, subglobose; br el, broadly ellipsoidal.
[c] Texture: rf, rough; sm, smooth

have clear wide furrows and distinctive crests. The convexes of ascospores are rough. ····· *E. amstelodami*

1. Mycelium color is diverse, white, yellow, orange, red etc. ····· 2
2. Colonies are yellow with cleistothecia and hyphae, and rare conidiation. Ascospores do not have clear ridges, but have clear wide furrows. ····· *E. tonophilum*
2. Colonies are diverse colors such as green, yellow, orange, red etc. ····· 3
3. Ascospores have clear flexible crests. Much larger growth on 37°C than 25°C on DG18 ····· *E. chevalieri*
3. Ascospores do not have clear crests. ····· 4
4. Ascospores have a narrow furrow which can be seen only as a trace by light microscopy. ····· *E. repens*
4. Ascospores have a clear wide furrow, and are usually larger than 5.0 μm. ····· 5
5. No growth or restricted growth on MEA. Smaller than 45 mm on ME20S. Ascospores are usually larger than 6.0 μm ····· *E. herbariorum*
5. Good growth on MEA (12-24 mm). Larger than 45 mm on ME20S. Colonies are usually red. ····· *E. rubrum*

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