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Ascospore Morphology and Ultrastructure of Species Assigned to the Genus *Lipomyces* Lodder et Kreger-van Rij

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Ascospores of *Lipomyces* species were examined by transmission electron microscopy. The following four types of ascospore morphology were found in replicas: (i) ascospores with longitudinal ridges; (ii) ascospores with irregular folds; (iii) ascospores with slightly undulating surfaces; and (iv) ascospores with smooth surfaces. In contrast, on the basis of ultrastructure of thin sections, only three types of ascospores were identified.

In 1974 Nieuwdorp et al. (4) studied the genus *Lipomyces* Lodder et Kreger-van Rij and recognized the following three types of ascospores: (i) smooth ascospores in *Lipomyces kononenkoae* Nieuwdorp et al. and *Lipomyces lipofer* Lodder et Kreger-van Rij; (ii) warty ascospores in *Lipomyces starkeyi* Lodder et Kreger-van Rij; and (iii) ridged ascospores in *Lipomyces tetrasporus* (Krassilnikov et al.) Nieuwdorp et al. These authors discerned the differences in ornamentation by transmission electron microscopy of serial sections. In addition to some biochemical characteristics, these workers subsequently used the ornamentation of ascospore walls in their key for identifying the *Lipomyces* species.

In 1975 Babjeva and Gorin (1) described a new species, *Lipomyces anomalus*. These authors reported that the ascospores of this species are spherical (rarely oval) and are surrounded by an "exosporium-like membrane," which was seen in transmission electron microscopic preparations of intact spores. A similar exosporium-like membrane was observed in replicas of the ascospores of *L. starkeyi*. The warts reported by Nieuwdorp et al. (4) on the spores of this species were not detected. In view of these different observations and because some isolates could not be identified by the key based on ascospore morphology and physiology, we began a study of ascospore morphology and ultrastructure as part of a revision of the genus *Lipomyces*. Light microscopy and transmission electron microscopy of ultrathin sections and replicas were used for comparison with the results reported previously.

MATERIALS AND METHODS

Strains. The yeast strains examined in this study and the conditions used for optimal sporulation are summarized in Table 1.

Light microscopy. The ornamentation of ascospore walls was observed by light microscopy with bright-field illumination, as well as with Nomarski differential interference contrast on specimens in water mounts.

Electron microscopy. The carbon replica technique of Bradley and Williams (3) was used to study the surfaces of the ascospores. If the asci did not release the ascospores, they were ruptured either mechanically with a Potter Elvehejm homogenizer or enzymatically by using Onozuka R-10 cellulase from *Trichoderma viride* (Serva, Feinbiochemie, Heidelberg, West Germany). The spores were prefixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0). A solution containing 3.5 g of potassium bichromate in 100 ml of 25% H_2SO_4 was used to dissolve the copper grid and spores within 2 h. The specimens were shadowed with platinum at an angle of 30° and examined with a Philips model EM 201 electron microscope at 60 kV.

For ultrathin sections the following two methods of fixation were used: (i) cells were prefixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 1 h and fixed with 1.5% aqueous $KMnO_4$ for 30 min at room temperature; and (ii) cells were fixed with 1.5% aqueous $KMnO_4$ for 30 min and postfixed with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.0) for 3 h at room temperature.

After fixation the cells were stained with 1.5% aqueous uranyl acetate during dehydration at the 50% ethanol step and finally embedded in Epon 812 or Spurr resin. Ultrathin sections were cut with a Reichert model OM-U4 ultramicrotome, poststained with Reynolds lead citrate for 1 min, and examined with a Philips model EM 201 electron microscope at 60 kV.

RESULTS AND DISCUSSION

Light microscopy. Three different types of ascospores were distinguished by light microscopy. The longitudinal striations on the oval ascospores of *L. tetrasporus* (Fig. 1) were easily recognized, just as Nieuwdorp et al. (4) reported and Babjeva and Meavad (2) showed previously. In all of the remaining *Lipomyces* species (including *Lipomyces* strains CBS 8064, CBS 8113, and CBS 8114) which could be induced to sporulate (Table 1), rough as well as smooth ascospores were observed in the same culture (Fig. 2), sometimes in the same ascus. The ascospores of the *L. starkeyi* strains and strain CBS 8064 were obviously more roughened than those of *L. kononenkoae*, *L. lipofer*, and strains CBS 8113 and CBS 8114. Some of these observations differ from those of Slooff (5), who did not observe ornamentation of the ascospores of *L. kononenkoae* and *L. lipofer* by light microscopy. This author did describe both smooth and ornamented ascospores of *L. starkeyi*.

Electron microscopy. Thorough fixation was often difficult to obtain due to the presence of lipid globules in the ascospores. The inner parts of the ascospores were frequently damaged (see Fig. 10) or lost (see Fig. 5) in sectioning. Four different types of mature ascospores were observed in replicas, and three types were observed in ultrathin sections.

(i) Ascospores with longitudinal ridges. Ascospores of this type were observed only in strains of *L. tetrasporus*. The

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TABLE 1. List of strains

Organism	Strain	Isolated from soil in:	Sporulation conditions		
			Medium in agar[a]	Incubation time (days)	Incubation temp (°C)
L. anomalus	CBS 6740[T][b]	U.S.S.R. (north taiga zone)	1/10 V8 (very few spores)	?	17
L. kononenkoae	CBS 2514[T]	Trinidad	1/10 V8; 1/10 YM; CM[a]	21	R.T.[c]
L. lipofer	CBS 944[T]	The Netherlands	YM	180	8
	CBS 2513	The Netherlands	1/10 V8	14	R.T.
	CBS 5841	?	1/10 V8	14	R.T.
	CBS 5842	Wales	1/10 V8; 1/10 YM	14–21	R.T.
L. starkeyi	CBS 1807[T]	United States	1/10 V8; 1/10 YM; CM	14	R.T.
	CBS 1809	United States	1/10 V8	26	R.T.
	CBS 2512	The Netherlands	Starkey ethanol	40	R.T.
	CBS 2516	South Africa	Starkey ethanol	40	R.T.
	CBS 6047	The Netherlands	1/10 YM; YM	180	8
L. tetrasporus	CBS 5910[T]	U.S.S.R.	1/10 YM; YM; CM	17	R.T.
	CBS 5910-1	U.S.S.R.	Ascospores not observed		
	CBS 1808	United States	Ascospores not observed		
	CBS 1810	Canada	YM	25	R.T.
	CBS 2511	Monaco	Ascospores not observed		
	CBS 5607	France	Ascospores not observed		
	CBS 5911	U.S.S.R	YM	21	R.T.
	CBS 6048	West Germany	YM	21	R.T.
	CBS 6049	U.S.S.R	YM	21	R.T.
	CBS 6050	U.S.S.R	YM	21	R.T.
	CBS 6051	U.S.S.R	Starkey ethanol	44	R.T.
Lipomyces species	CBS 8064[d]		1/10 YM; 1/10 V8; YM	28	R.T.
	CBS 8113	South Africa	1/10 YM; YM	21–28	R.T.
	CBS 8114	South Africa	1/10 YM; YM	21–28	R.T.

[a] 1/10 V8, V8 Vegetable Juice medium (6) diluted 1:10. (CM, corn meal; YM, yeast extract-malt extract [Difco]).
[b] T = Type strain.
[c] R.T., Room temperature.
[d] Isolated from a lemon in France.

observations of Nieuwdorp et al. (4) were confirmed by the replicas of mature ascospores. Not every ridge extended the full length of the spore (Fig. 3), and the ridges did not converge at one point on the pole; in some cases the ridges extended over the pole (Fig. 4).

In ultrathin sections the wall of each mature ascospore had

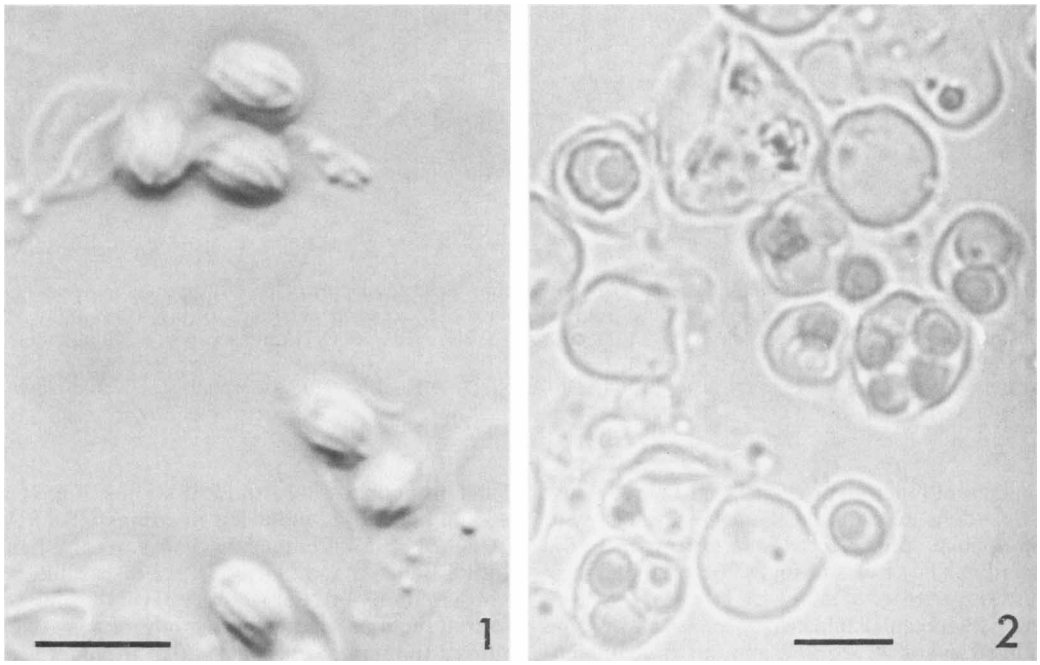

FIG. 1. *L. tetrasporus* CBS 6050 ascospores showing longitudinal striation. Light microscopy with differential interference contrast. Bar = 5 μm.

FIG. 2. Rough and smooth ascospores of *L. starkeyi* CBS 1807 in separate asci. Light microscopy with bright-field illumination. Bar = 5 μm.

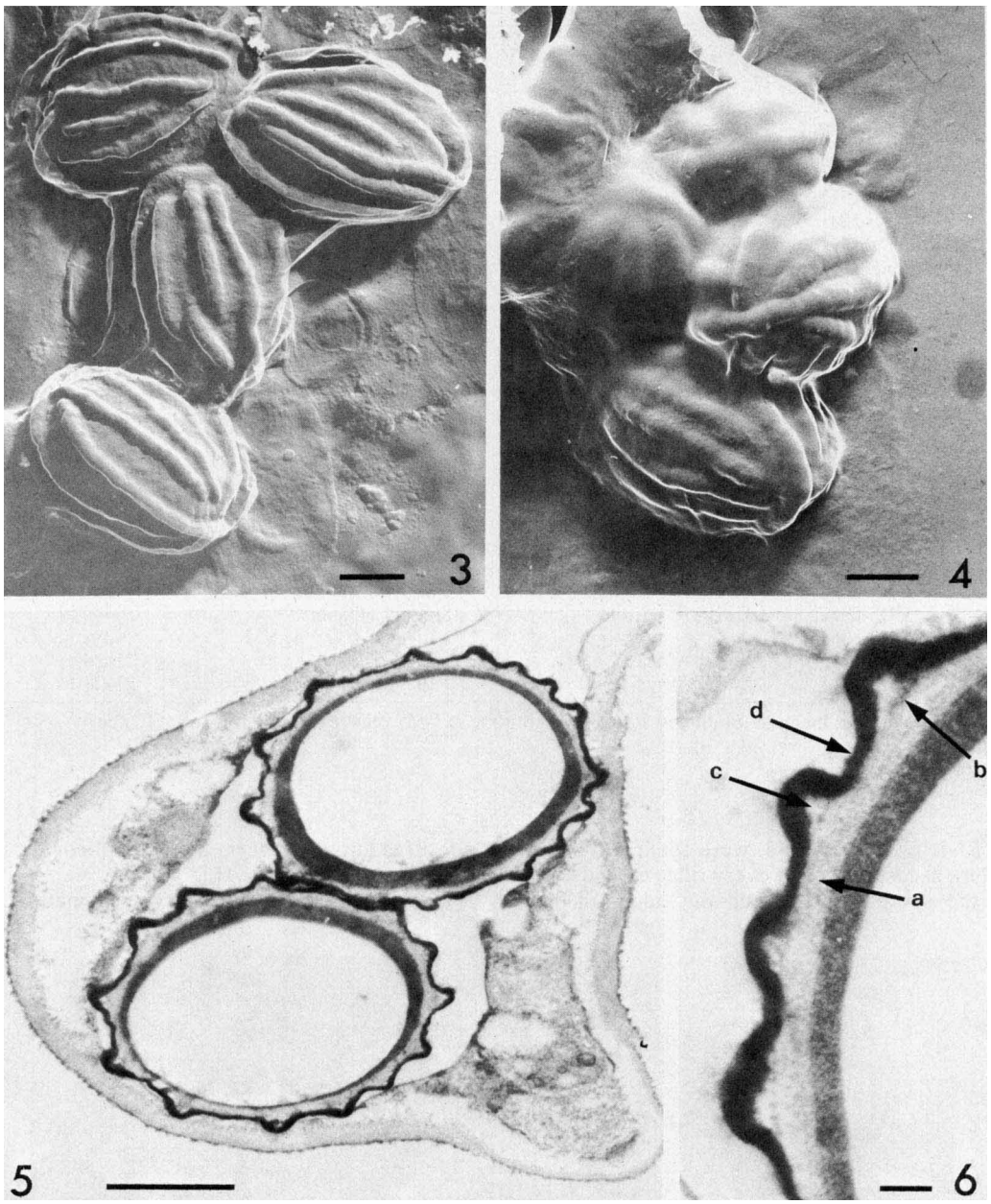

FIG. 3. Transmission electron micrograph of a replica from ascospores of *L. tetrasporus* CBS 5911 printed in negative. Bar = 1 μm.
FIG. 4. Transmission electron micrograph of a replica from ascospores of *L. tetrasporus* CBS 5910^T printed in negative. Bar = 1 μm.
FIG. 5. Transmission electron micrograph of a *L. tetrasporus* CBS 5910^T ascus with ascospores. Glutaraldehyde-potassium permanganate fixation. Bar = 1 μm.
FIG. 6. Transmission electron micrograph of a portion of a *L. tetrasporus* CBS 5910^T ascospore wall. Glutaraldehyde-potassium permanganate fixation. Bar = 0.1 μm.

an electron-transparent inner layer (Fig. 5 and Fig. 6, arrow a) surrounded by a delicate, thin, dark line (Fig. 6, arrow b) and an electron-opaque outer layer (Fig. 6, arrow d). In the protuberances a fourth layer was observed between the dark line and the outer layer (Fig. 6, arrow c).

(ii) Ascospores with irregular folds. Ascospores of this type were observed in strains of *L. starkeyi* and the three unidentified *Lipomyces* strains (strains CBS 8064, CBS 8113, and CBS 8114). The replicas of mature ascospores demonstrated that the ornamentation was due to the presence of irregular folds (exosporium-like membrane [1]), which were distinct and pronounced in *L. starkeyi* strains (Fig. 7) and strain CBS 8064, but less pronounced in strains CBS 8113 (Fig. 9) and CBS 8114, a difference also observed by light microscopy. Like Babjeva and Gorin (1), we also could not confirm the observations of Nieuwdorp et al. (4), who concluded from serial sections that the ascospores of *L. starkeyi* were warty. From the replicas it is clear that if only a few sections of a series are examined, it is easy to misinterpret the irregular folds as warts as Nieuwdorp et al. (4) did. The light microscopic observation of the presence of both smooth and ornamented ascospores was confirmed by examining ul-

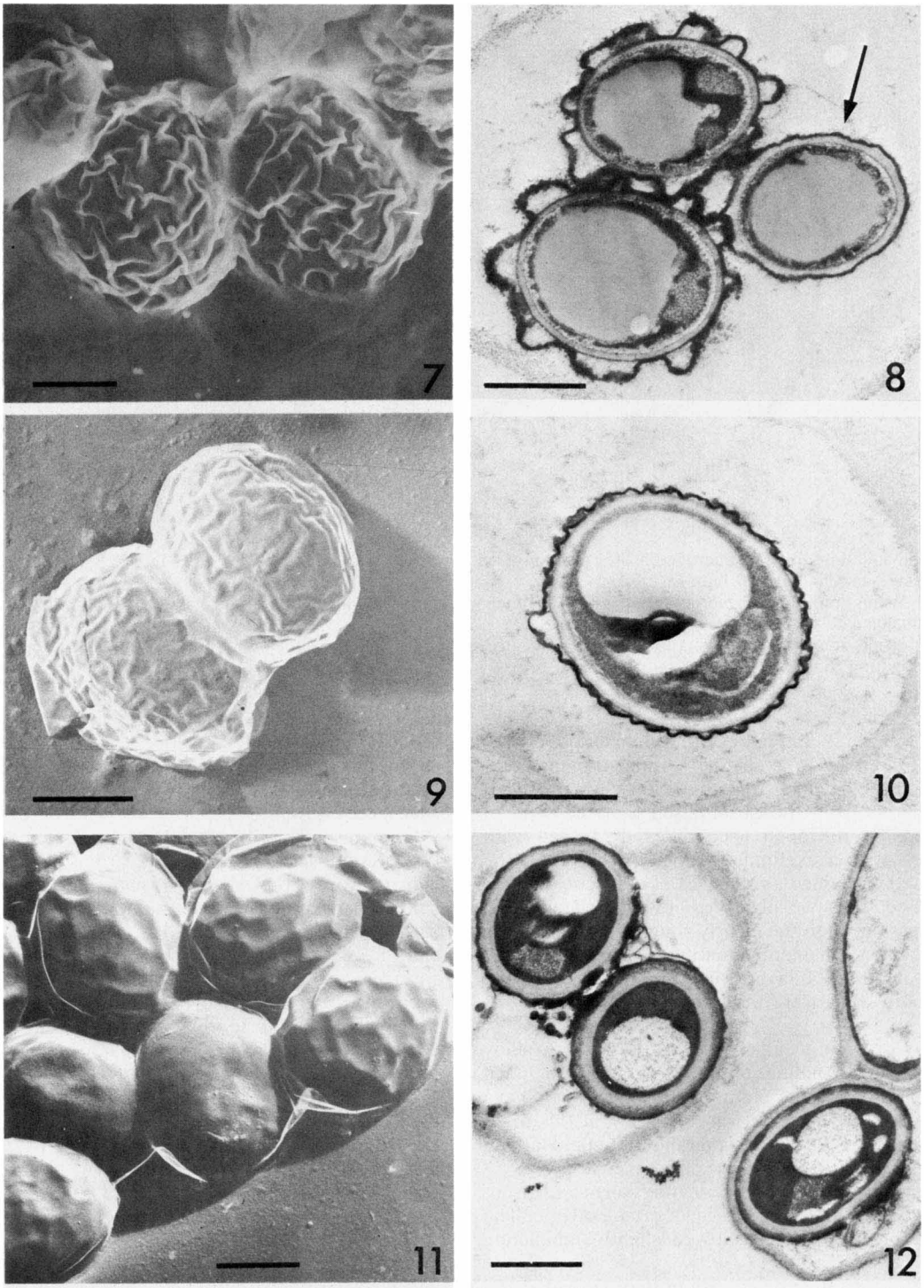

FIG. 7. Transmission electron micrograph of a replica from ascospores of *L. starkeyi* CBS 1807^T printed in negative. Bar = 1 μm.

FIG. 8. Transmission electron micrograph of a *Lipomyces* species strain CBS 8064 ascus with rough and almost smooth (arrow) ascospores. Potassium permanganate-osmium tetroxide fixation. Bar = 1 μm.

FIG. 9. Transmission electron micrograph of a replica of ascospores of *Lipomyces* species strain CBS 8113 printed in negative. Bar = 1 μm.

FIG. 10. Transmission electron micrograph of an ascospore of *Lipomyces* species strain CBS 8114. Glutaraldehyde-potassium permanganate fixation. Bar = 1 μm.

FIG. 11. Transmission electron micrograph of a replica of ascospores of *L. kononenkoae* CBS 2514^T printed in negative. Bar = 1 μm.

FIG. 12. Transmission electron micrograph of asci with ascospores of *L. kononenkoae* CBS 2514^T. Glutaraldehyde-potassium permanganate fixation. Bar = 1 μm.

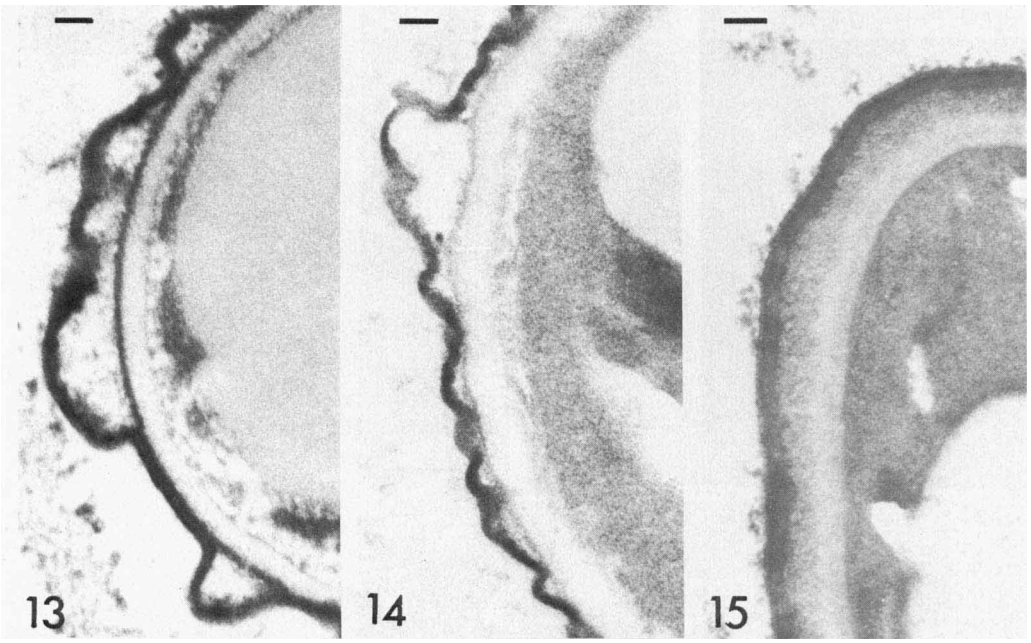

FIG. 13. Transmission electron micrograph of a portion of an ascospore wall of *L. starkeyi* CBS 1807^T. Potassium permanganate-osmium tetroxide fixation. Bar = 0.1 μm.

FIG. 14. Transmission electron micrograph of a portion of an ascospore wall of *Lipomyces* species strain CBS 8114. Glutaraldehyde-potassium permanganate fixation. Bar = 0.1 μm.

FIG. 15. Transmission electron micrograph of a portion of an ascospore wall of *L. kononenkoae* CBS 2514^T. Glutaraldehyde-potassium permanganate fixation. Bar = 0.1 μm.

trathin sections (Fig. 8). We believe that the smooth spores visible by light microscopy as distinct entities are immature spores on which the irregular folds have not yet developed. We concluded this from the observation that the ratio of smooth ascospores to rough ascospores decreased with time; this observation also eliminated the possibility that the strains examined contained ascospores of different shapes.

In ultrathin sections we observed no difference in the spore wall layers between the clearly roughened ascospores (Fig. 13) and the less ornamented mature ascospores (Fig. 10 and 14). The number of layers and the structure of the mature ascospore walls were the same as in *L. tetrasporus* (Fig. 6).

L. anomalus did not yield enough ascospores to allow electron microscopic examination. However, we concluded from the description and the pictures published by Babjeva and Gorin (1) that the ascospore morphology of this organism resembles that of *L. starkeyi* and the three unidentified *Lipomyces* strains.

(iii) Ascospores with slightly undulating surfaces. Ascospores of this type were observed in *L. kononenkoae*. The replicas of mature ascospores showed slightly undulating surfaces (Fig. 11).

In ultrathin sections the presence of both ornamented and smooth ascospores as observed by light microscopy was confirmed (Fig. 12). As in *L. starkeyi* and the three unidentified *Lipomyces* strains, we believe that the smooth ascospores are immature and disappear as the culture ages. The wall of each mature ascospore consisted of a thick electron-transparent inner layer that became progressively dense toward the surface and an electron-opaque outer layer (Fig. 15). In this respect mature ascospores of *L. kononenkoae* differ from mature ascospores of strains CBS 8113 and CBS 8114 (Fig. 14) more obviously than the gross morphology in replicas suggests. The presence of slightly ornamented ascospores in *L. kononenkoae* was not mentioned by Nieuwdorp et al. (4). In our opinion, these authors examined immature ascospores only.

(iv Ascospores with smooth surfaces. Ascospores of this type were observed in *L. lipofer* strains. The observation by light microscopy of slightly ornamented ascospores beside smooth ascospores was not supported by the transmission electron microscopy studies. In ultrathin sections (Fig. 19) and in replicas (Fig. 16) only ascospores with smooth surfaces were found. In the strains studied, ornamented ascospores were never observed as the cultures aged. The irregularities on the spore wall, as observed by light microscopy, were cytoplasmic remnants, which were also often observed in ultrathin sections (Fig. 17).

Ultrastructural sections of mature ascospores showed a thick electron-transparent inner layer and a fairly thick electron-opaque outer layer surrounded by a thin, fringed membrane (Fig. 18).

Our observations of this type of ascospore morphology confirm the observations of Nieuwdorp et al. (4).

Our observations of the ornamentation and ultrastructure of *Lipomyces* ascospores as observed by light microscopy and electron microscopy are summarized in Table 2. The following four types of mature ascospore morphology were distinguished: (i) ascospores with longitudinal ridges; (ii) ascospores with irregular folds; (iii) ascospores with undulating surfaces; and (iv) ascospores with smooth surfaces. We observed that smooth ascospores, although visible by light microscopy as distinct, solid entities, do not always represent mature ascospores, but are instead immature spores in some species. We also noticed that in most species, ascospore morphology could not be determined unambiguously by light microscopy. In only one case could morphology be

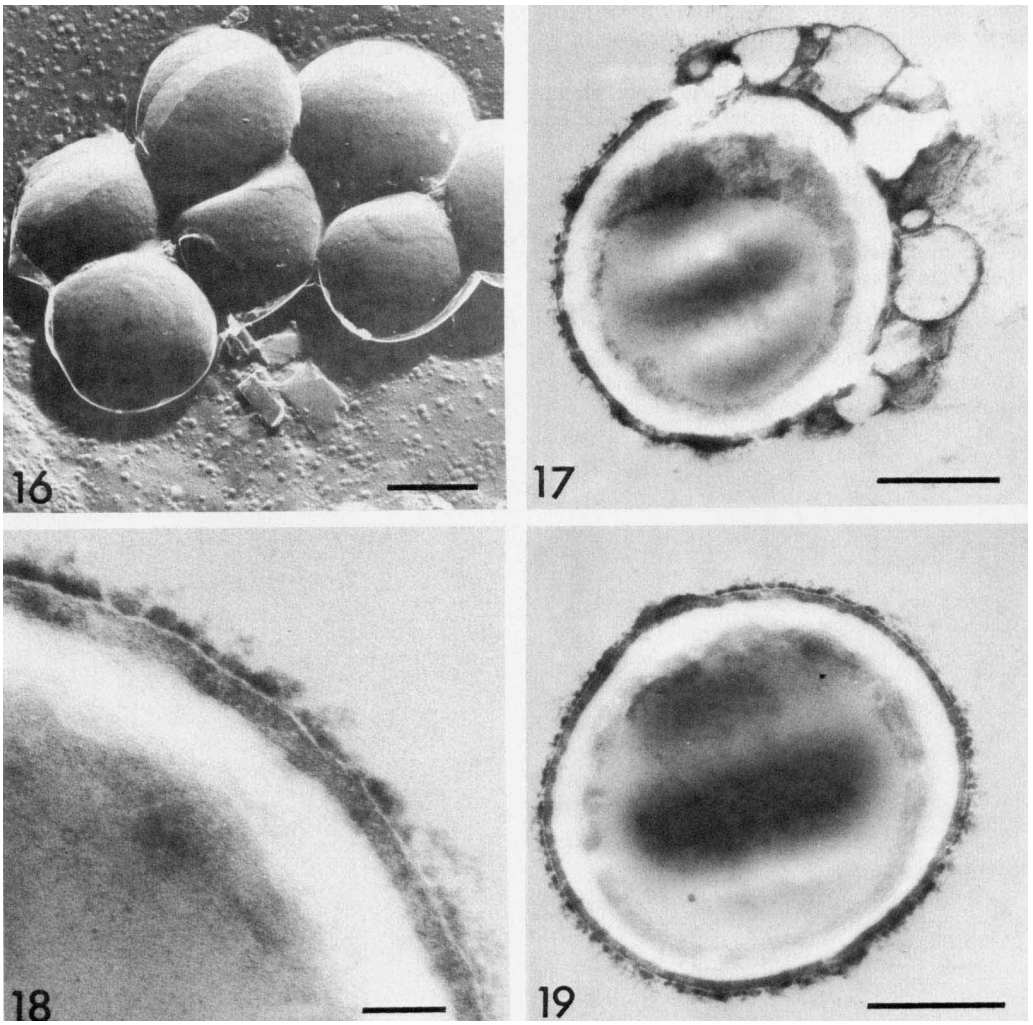

FIG. 16. Transmission electron micrograph of a replica of ascospores of *L. lipofer* CBS 5842 printed in negative. Bar = 1 μm.

FIG. 17. Transmission electron micrograph of an ascospore of *L. lipofer* CBS 5842. Potassium permanganate-osmium tetroxide fixation. Bar = 0.5 μm.

FIG. 18. Transmission electron micrograph of a portion of an ascospore wall of *L. lipofer* CBS 5842. Potassium permanganate-osmium tetroxide fixation. Bar = 0.1 μm.

FIG. 19. Transmission electron micrograph of an ascospore of *L. lipofer* CBS 5842. Potassium permanganate-osmium tetroxide fixation. Bar = 0.5 μm.

determined unequivocally (namely, when the ascospores had ridges). In all other cases ascospore morphology had to be established by electron microscopy. However, to avoid the use of electron microscopy for identification as done by Nieuwdorp et al. (4), we plan to look for more convenient tests during a revision of the genus. Evidence from thin sections suggests that the cell walls of mature ascospores can be divided into three groups. The walls of the spores consist of: (i) two layers, as in *L. kononenkoae*; (ii) three layers, as in *L. lipofer*; or (iii) four layers, as in *L. tetrasporus, L. starkeyi*, and the three unidentified *Lipomyces* strains.

TABLE 2. Ornamentation and ultrastructure of ascospores in the genus *Lipomyces*

Organism(s)	Ornamentation as determined by:		Ultrastructure (no. of cell wall layers in mature ascospores)
	Light microscopy	Transmission electron microscopy	
L. tetrasporus	Ridges (Fig. 1)	Ridges (Fig. 3 and 4)	4 (Fig. 6)
L. starkeyi and *Lipomyces* sp. strain CBS 8064	Smooth, warty (Fig. 2)	Smooth (Fig. 8), irregular folds (Fig. 7 and 8)	4 (Fig. 13)
Lipomyces sp. strains CBS 8113 and CBS 8114	Smooth, slightly ornamented	Smooth, irregular folds (Fig. 9 and 10)	4 (Fig. 14)
L. kononenkoae	Smooth, slightly ornamented	Smooth, slightly undulating (Fig. 11 and 12)	2 (Fig. 15)
L. lipofer	Smooth, slightly ornamented	Smooth (Fig. 16)	3 (Fig. 17)

The three unidentified *Lipomyces* strains, whose ascospore morphology and ultrastructure resemble those of *L. starkeyi*, differ from this species in five to seven physiological characteristics. Physiologically, these strains are similar to *L. kononenkoae*. However, before new taxa are erected, more detailed studies of morphology, physiology, life cycles, guanine-plus-cytosine contents of deoxyribonucleic acids, and, in particular, deoxyribonucleic acid-deoxyribonucleic acid homology should be performed to evaluate the taxonomic value of the morphology and ultrastructure of the ascospores in the genus *Lipomyces*.

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