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Keratinolysis and its morphological expression in hair digestion by airborne fungi

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Abstract. The morphological expression of keratinolysis in fungi isolated from the air of Torino (98 isolates belonging to 36 species) was studied. Light microscopy on whole material and on semithin sections, as well as scanning electron microscopy was used. There were 19 keratinolytically active species, with seven in the genus *Chrysosporium* (*C. indicum, C. keratinophilum, C. pannicola, C. tropicum,* C. an. *Arthroderma cuniculi,* C. an. *Pectinotrichum llanense,* C. an. *Renispora flavissima*), four in the genus *Malbranchea* (*M. arcuata, M. fulva, M. sulphurea,* M. st. *Uncinocarpus reesii*), and three in the genus *Trichophyton* (*T. mentagrophytes, T. rubrum, T. terrestre*). In addition there were *Aphanoascus fulvescens, Beauveria bassiana, Geomyces pannorum* v. *pannorum, Gymnoascus umbrinus* and *Myceliophthora vellerea.* Most of these species were capable of developing structures related to surface erosion and radial penetration contemporaneously. However *Gymnoascus umbrinus, Myceliophthora vellerea,* an isolate of *C. indicum, C. tropicum* and *Trichophyton mentagrophytes* demonstrated only surface erosion. Different isolates of one species can vary in their production of invasive structures and in degree of keratinolytic activity. Thus such activity, like many biochemical activities of fungi, does not appear to be a constant or rigorously species-specific character.

Key words: Airborne fungi, *Chrysosporium* and allied genera, Hyphomycetes with arthroconidia, Keratinolytic activity, *Malbranchea, Trichophyton*

Introduction

There are two major reasons for the current interest in keratinolytic fungi. First, they play an important ecological role in decomposing cornified residues [1, 2]. For this reason the selection of particularly active strains may be, or could become, useful in managing heavily populated areas, either for introduction into natural habitats, or especially for installations designed for processing garbage or purifying waste water. Second, many keratinolytic fungi have properties in common with dermatophytes, and some, although rarely, can probably cause human infections [3]. An understanding of the way in which these two groups of fungi attack keratinic substrates could shed fresh light on their physiologic and pathogenic similarities [4] and the mechanisms that regulate pathogenicity. The capacity to digest α-keratins in fact presupposes unusual metabolic characteristics [5, 6], since these substances are very resistant due to their high cystine content.

Keratinolytic fungi, whether dermatophytes or not, make contact with keratinic material, organize and also possibly alter their hyphae, and completely digest the substrate. All these processes require closer investigation, including certain biochemical aspects.

In the present work we have studied the morphological expression of keratinolysis in fungi isolated from the air of Torino. We have developed methods of demonstrating keratinolysis, and have attempted to define the mode of attack on human hair in vitro, including study of lytic spaces and specialized fungal organs. Light microscopy has been used on whole material and on semithin sections, as well as scanning electron microscopy.

Materials and methods

Organisms and culture conditions. We examined 98 isolates belonging to 36 species, both anamorphic and teleomorphic, as follows:

Aphanoascus fulvescens (Cooke) Apinis
Arthrographis cuboidea (Sacc. & Ellis) Sigler
Beauveria bassiana Vuill.
Chrysosporium carmichaelii van Oorschot
C. indicum (Randhawa & Sandhu) Garg
C. keratinophilum D. Frey ex Carmichael
C. pannicola (Corda) van Oorschot & Stalpers
C. pseudomerdarium van Oorschot
C. queenslandicum Apinis & Rees
C. sulfureum (Fiedl.) van Oorschot & Samson
C. tropicum Carmichael
C. xerophilum Pitt
C. an. *Arthroderma cuniculi* Dawson
C. an. *Gymnoascus demonbreunii* Ajello & Cheng
C. an. *Pectinotrichum llanense* Varsavsky & Orr
C. an. *Renispora flavissima* Sigler et al.
Cladobotryum varium Nees ex Steudel
Geomyces pannorum (Link) Sigler & Carmichael *v. pannorum*
G. pannorum (Link) Sigler & Carmichael *v. asperulatus* (Sigler & Carmichael) van Oorschot
Gymnoascus reessii Baran.
G. umbrinus Boudier
Malbranchea arcuata Sigler & Carmichael
M. chrysosporoidea Sigler & Carmichael
M. flava Sigler & Carmichael
M. fulva Sigler & Carmichael
M. gypsea Sigler & Carmichael
M. pulchella Saccardo & Penzig
M. sulphurea (Miehe) Sigler & Carmichael
M. st. *Uncinocarpus reesii* Sigler & Orr
Myceliophthora lutea Cost.
M. vellerea (Sacc. & Speg.) van Oorschot
Myxotrichum deflexum Bérkeley
Ovadendron sulphureo-ochraceum (v. Beyma) Sigler & Carmichael
Trichophyton mentagrophytes (Robin) Blanchard
T. rubrum (Castellani) Sab.
T. terrestre Durie & Frey

All isolates came from outdoor air of Torino (Italy) and were collected at a height of about 2 m, in three areas with differing urban and ecological characteristics [3, 7], over a period of one year. The collector was a one stage volumetric sieve sampler (SAS, Pool Bioanalyse Italiana) and the collection medium was Dermasel agar (Oxoid) supplemented with 0.4 g l^{-1} cycloheximide and 50 mg l^{-1} chloramphenicol (Oxoid antibiotic supplement for Dermasel agar – SR 75). After isolations, cultures were stored on malt agar at 6–7 °C. For testing, they were transferred to malt agar or PYE (phytone yeast extract) agar and incubated at 24 °C. This second culture medium stimulates growth and fruiting, which, after storage of the culture at low temperature, sometimes appear to be slowed or modified.

Keratinolysis test. The isolates were grown on a soil-hair substrate using the technique of English [8]. According to this author the soil-hair system furnishes keratinic material as well as additional nutrients that possibly play an important role in stimulating differentiation of specialized fungal organs; in addition, the culture conditions are closer to those found in nature, so that the information obtained has greater ecological relevance.

Petri dishes 5 cm in diameter were part-filled with garden soil and twice autoclaved. Pieces of autoclaved hair about 1 cm long were then added; the hair came from a blond child. The preparations were then inoculated with 1 ml of aqueous spore suspension and 1 ml of water. This suspension was obtained by washing a good fruiting culture on PYE with 10 ml of sterile water. The preparations were then incubated at 24 °C for 20 days. Three replicates were inoculated for each isolate, to control for reproducibility and growth rate. On about the tenth day of incubation a further 1ml of sterile water was added to each dish.

Light microscopy. (1) *Observations on entire fungus-hair units.* Hairs supporting fungal growth were sampled every 5th day for 20 days. Fungal growth was evaluated macroscopically or using a stereomicroscope (Wild M3) equipped with fibre optics. Samples for microscopy were taken from two of the three replicate plates, the third being left intact for observation of growth. Hairs were mounted in lactophenol cotton blue [9] for examination. Results were interpreted in light of the model proposed by Filipello Marchisio [1], and this procedure also served to test the model. The term surface erosion indicates the progressive destruction of the hair from the exterior inwards; this may occur either uniformly along the length of the hair or in localized areas forming more or less extensive pockets. The term radial penetration indicates random attack on the hair by more or less specialized hyphae that penetrate the hair at right angles to the surface. The term boring hypha and perforating organ are used in the sense of English [1, 8]. Wider boring hypha indicates structures intermediate in diameter between boring hyphae and perforating

Table 1. Keratinolytic activity in vitro

Species and isolate	G	F	SE		RP				I
			u	p	bh	sbh	wbh	po	
Aphanoascus fulvescens									
10.1.C/11.6	3	+	+	+	+	+			3
3.1.C/11.4	4		+	+	+				4
10.1.C/18.2	2	+	+		+	+			3
12.1.V/19.25	4	+	+		+	+			3–4
5.1.V/86.A1	4	+	+	+	+	+		+	4
Arthrographis cuboidea									
2.1.C/2.46	1	+	+						0–1
Beauveria bassiana									
8.1.C/20.25	1	+			+				1
8.1.P/14.26	1	+			+				1
8.1.C/7.25	1				+				1
Chrysosporium carmichaelii									
11.1.C/8.10	1	+	+						1
10.1.C/12.25	1	+	+						1
10.1.V/7.25									0
6.1.P/8.2F									0
Chrysosporium indicum									
3.1.C/3.5	4	+	+	+					4
3.1.C/3.11	4	+	+	+	+		+		4
Chrysosporium keratinophilum									
10.1.P/15.2B	4	+	+	+	+	+	+		4
3.1.P/1.6	4	+	+	+	+	+	+		4
9.1.V/3.6	4	+	+	+	+	+	+		4
Chrysosporium pannicola									
3.2.C/7.17	4	+	+	+	+	+	+		4
Chrysosporium pseudomerdarium									
4.1.V/22.17	1		+						0–1
Chrysosporium queenslandicum									
10.1.C/2.4	1		+						0–1
Chrysosporium sulfureum									
11.1.V/6.2	1	+	+						1
9.1.V/17.25	1	+	+						1
10.1.C/8.24	1	+	+						1
2.1.V/14.6	2	+	+						1
Chrysosporium tropicum									
2.1.V/6.6	4	+	+	+	+	+			4
3.1.C/4.6	4	+	+	+	+	+	+		4
10.1.C/12.11	3	+	+						3

organs. Swollen boring hyphae are structures that are similar to boring hyphae when penetrating the outer cortex, but dilated in a series of balloons on reaching what are probably less compact regions of the hair. Intensity of attack on the hair was estimated on a scale of 0 to 4: 0, no attack; 0–1, light attack on the cuticle; 1, moderate attack on the cuticle and/or rare formation of boring hyphae; 2, attack on cuticle and cortex, with about 20% destruction of the hair; 3, as above, with about 50% destruction of the hair; 4, as above, with about 80% destruction.

Table 1. Continued.

Species and isolate	G	F	SE		RP				I
			u	p	bh	sbh	wbh	po	
Chrysosporium xerophilum									
3.2.V/11.6	1	+	+						1
Chrysosporium an. *Arthroderma cuniculi*									
6.1.C/21.46	4	+	+	+	+	+	+		3
Chrysosporium an. *Gymnoascus demonbreunii*									
7.1.P/19.5	1		+						0–1
3.1.C/11.3	1		+						0–1
Chrysosporium an. *Pectinotrichum llanense*									
4.1.V/8.3	4	+	+					+	4
4.1.V/8.4	4	+	+	+				+	4
Chrysosporium an. *Renispora flavissima*									
10.1.C/8.75	4	+	+	+	+			+	4
Cladobotryum varium									
10.1.C/9.6	1		+						0
10.1.C/4.73	1		+						0
10.1.C/9.73	1		+						0–1
Geomyces pannorum v. *pannorum*									
7.1.C/5.27	2		+		+				1
6.1.V/6.6	1		+						1
3.1.C/6.4	1		+						1
3.1.V/4.23	1		+						0–1
6.1.P/19.25	1		+						1
11.1.P/8.14	2	+	+						1
9.1.P/21.14	1		+						1
3.1.V/9.17	1		+						0–1
9.1.P/21.6	2	+	+		+				1
3.1.C/9.13	2		+		+				1
3.1.C/4.12	2	+	+						1
3.1.C/5.7	1		+		+				0–1
Geomyces pannorum v. *asperulatus*									
11.1.C/12.27	1		+						0–1
Gymnoascus reessii									
2.1.V/24.62	3	+	+						0-1
3.1.C/8.14	2		+						0–1
3.1.P/22.25	1		+						0–1
3.1.C/3.14	2		+						0–1
7.1.C/15.14									0
Gymnoascus umbrinus									
1.1.V/4.27	3	+	+						2

(2) *Observations on semithin sections of fungus-hair units.* Pieces of hair 1 mm long, supporting fungal growth, were fixed for 3 h in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7, then washed twice in the same buffer. They were then postfixed for 3 hours in 1% osmium tetroxide, again washed twice in cacodylate buffer, and dehydrated in an ethanol series, followed by two passages in 100% dry acetone. They were then embedded in Durcupan ACM resin.

Table 1. Continued.

Species and isolate	G	F	SE		RP				I
			u	p	bh	sbh	wbh	po	
Malbranchea arcuata									
10.1.C/22.62	1		+		+				1
1.1.V/12.11	1		+						1
1.1.V/14.49	1		+						1
2.1.C/11.11	1		+						1
1.1.V/12.10	1		+						1
10.1.P/4.46	1		+						1
4.1.C/13.8	1		+						1
10.1.P/6.6	2		+						1
Malbranchea chrysosporoidea									
11.1.V/8.15	2		+						0–1
3.2.C/7.39	1	+	+						0–1
4.1.V/4.10	1		+						0–1
12.1.C/13.12	1		+						0–1
Malbranchea flava									
1.1.V/17.6	1	+	+						0–1
Malbranchea fulva									
10.1.C/7.11	1		+		+				1
2.1.C/21.39	1		+						0–1
Malbranchea gypsea									
3.2.C/19.14	1	+	+						1
Malbranchea pulchella									
1.1.P/7.6	1		+						0–1
4.1.C/22.6	1		+						1
11.1.V/17.25	1		+						1
10.1.V/1A	1		+						0–1
Malbranchea sulphurea									
1.1.V/13.11	1	+	+	+	+				1
1.1.V/3.25	2	+	+	+	+				1
12.1.C/14.2	1	+	+	+	+				1
Malbranchea st. *Uncinocarpus reesii*									
2.1.P/4.2	4	+	+	+	+		+	+	4
1.1.P/11.11	4	+	+	+	+	+		+	4
1.1.C/12.17	4	+	+	+	+	+		+	4
Myceliophthora lutea									
5.1.V/18.14	2	+	+						1

Sections about 1 μm thick were stained in toluidine blue or using the periodic acid – Schiff (PAS) reaction [10] and examined by light microscopy.

The above method was used for only some species, and was designed to examine different modes of attack (Table 1): *Chrysosporium indicum, C. keratinophilum, C.* an. *Arthroderma cuniculi, C.* an. *Pectinotrichum Ilanense, C.* an. *Renispora flavissima, Malbranchea* st. *Uncinocarpus reesii* were capable of developing structures related to surface erosion and radial penetration; *Myceliophthora vellerea* only showed surface erosion either uniform or in pockets; *Beauveria bassiana* only developed rare boring hyphae. Samples relating to those species attacking rapidly were taken from day 3 of incubation (*Chrysosporium indicum, C. keratinophilum, C.* an. *Pectinotrichum Ilanense, C.* an. *Renispora flavissima, Malbranchea* st. *Uncinocarpus reesii*), the sam-

Table 1. Continued.

Species and isolate	G	F	SE		RP				I
			u	p	bh	sbh	wbh	po	
Myceliophthora vellerea									
11.1.C/9.25	4	+	+	+					3
11.1.V/7.2A	4	+	+	+					3
Myxotrichum deflexum									
9.1.V/12	1		+						1
3.2.C/7.11	1		+						1
3.2.C/4.17	1		+						1
3.2.C/7.11B	1		+						1
7.1.C/4.53	1		+						1
Ovadendron sulphureo-ochraceum									
2.1.V/22.6	1	+	+						1
Trichophyton mentagrophytes									
4.1.C/16.6	2		+						1
2.1.V/11.5	3		+					+	4
Trichophyton rubrum									
6.1.C/21.11	4	+	+	+				+	4
6.1.C/21.2	3	+	+	+				+	4
Trichophyton terrestre									
10.1.C/20.4	4	+	+	+	+			+	4
10.1.P/6.6	4	+	+	+			+	+	4
2.1.C/20.4	2	+	+	+			+	+	2

G, growth; F, fruiting; SE, surface erosion; RP, radial penetration; I, intensity of attack on the hair; u, uniform erosion; p, erosion in localized areas (pockets); bh, boring hypha; sbh, swollen boring hypha; wbh, wider boring hypha; po, perforating organ.

Growth: 1, poor, visible only with the stereomicroscope; 2, moderate; 3, good; 4, abundant.

Intensity of attack: 0, none; 0–1, light attack on the cuticle; 1, moderate attack on the cuticle and/or rare formation of boring hyphae; 2, attack on cuticle and cortex, with about 20% destruction of the hair; 3, as above, with about 50% destruction of the hair; 4, as above, with about 80% destruction.

ples relating to those species attacking more slowly being taken on day 5 (*C.* an. *Arthroderma cuniculi, Myceliophthora vellerea*) or on day 10 (*Beauveria bassiana*).

Scanning electron microscopy (SEM). Samples of the same preparations studied in semithin sections were prepared, fixed and dehydrated as above. They were then processed in a K 850 Critical Point Drier and coated with gold in a Bio-Rad SC Sputter Coater. Before metal coating some samples were frozen in liquid N_2 and split transversely. Other samples embedded in resin were sectioned with an ultramicrotome until the structure of interest was reached. The surface resin was then removed with sodium methylate (2.5 g in 25 ml methanol plus 25 ml benzene) [11]. They were examined in an ETEC Autoscan SEM at 20 kV.

Results

Keratinolysis tests and light microscopy of entire fungus-hair units. Table 1 summarizes the final data (after 20 days' incubation) on growth, fruiting, method of attack on the hair, and its intensity estimated on a scale of 0 to 4. The values obtained were taken as a guide to the efficiency of keratinolysis. All species which eroded the hair surface or penetrated radially, clearly evidencing that the keratin molecule was being digested, were considered as possessing keratinolytic activity and were rated from

2 to 4. In addition *Beauveria bassiana, Geomyces pannorum* v. *pannorum, Malbranchea arcuata, M. fulva* and *M. sulphurea* were capable of producing boring hyphae, although they were rated at intensity level 1. The keratinolytic capacity of these delicate structures was also demonstrated gravimetrically by Böhme & Ziegler [12] and confirmed ultrastructurally by Fusconi & Filipello Marchisio [4], although originally English [13] had supposed them capable of only physical penetration of the substrate.

The other isolates, assigned attack intensity ratings of 0–1/1, showed some sign of altering the cuticle (partial lifting of the scales and permeability to lactophenol cotton blue). This we attribute, however, only to an ability to utilize the material cementing the scales, or to a production of aspecific proteases. Such proteases could enable the fungus to reach as far as the endocuticle, formed from modified cytoplasmic remains [14], but not to attack the keratin molecules present at other levels of the cuticular scales and in the cortex [14]. This question can only however be resolved by TEM ultrastructural analysis of the fungus-hair system.

Table 1 shows that different isolates of one species can vary in their production of invasive structures. For example, only one isolate of *Aphanoascus fulvescens* and *Trichophyton mentagrophytes* produced perforating organs; only some isolates of *Chrysosporium indicum, C. tropicum, Geomyces pannorum* v. *pannorum, Malbranchea arcuata* and *M. fulva* produced some kind of radial penetration and further only two isolates of *C. charmichaelii* were able to grow, fruit and alter the cuticle of the hair. The degree of keratinolytic activity can vary too. For example different isolates of *Aphanoascus fulvescens* and *Chrysosporium tropicum* were rated 3 or 4 and 2 or 4 those of *T. terrestre*.

Figure 1 shows examples of lifting of the cuticle (a), uniform surface erosion (d) and pockets (b,c), of radial penetration by boring hyphae (e), swollen boring hyphae (f), wider boring hyphae (g) and perforating organs (h). Figure 1i also shows advanced stages of demolition of the hair, with confluence of many lytic areas, which are difficult to account, for using the model of Filipello Marchisio [1].

Light microscopy of semithin sections of fungus-hair units. This method of investigation permitted improved resolution of the fungal structures involved in attack on the hair, their relationship to the matrix, and the sequence of events involved in hair demolition. A tentative reconstruction of the invasion model of the hair, through different forms of attack, is given. References to figures have to be considered mere examples of a general pattern within the limits of the forms of attack singled out and summarized in Table 1. These latter appear to develop similarly, irrespective of the involved fungal species. However not all forms could be seen on sectioned hair.

In the first stages of invasion, hyphae penetrate beneath cuticular scales, causing lifting of the cuticle (Fig. 2a). The hyphae appear normal or may dilate and form short branches, giving rise to 'palm of the hand' structures described by English [17]. In our opinion these are not very different from the hyphopodia that plant pathogenic fungi may produce during penetration of intact plant surfaces. Digestion of the cuticle seems therefore to be mediated by hyphae of these two types, that slowly attack from the inside towards the outside of the scales.

Similarly, the outer layers of the cortex are also enzymatically attacked by such hyphae. In this case, digestion appears to progress more rapidly; the cortex loses its compact structure, and the cells and macrofibrillar bundles of keratin become separated, at a time when the cuticular scales are still intact or only partially digested (Fig. 2a, d).

On the cortex, the hyphae involved in attack on the cuticle develop further, giving rise to flattened, branched fronds of mycelium, called by English [17] eroding mycelium. This, together with other hyphae that retain their normal appearance, form a more or less extensive hyphal mantel around the hair, and this is responsible for the so-called uniform surface erosion (Fig. 2b, c). The eroding mycelium and perhaps also the simpler 'palm of the hand' structures can cause erosion pockets, first by means of large cells 4-5 μm in diameter (Fig. 2d) that slowly create cavities of various sizes. Later the initial cells develop stubby branches parallel to the long axis of the hair, that penetrate between the cells of the cortex (Fig. 2e). Alternatively, a more or less compact pseudoparenchyma develops, and this can extend considerably and may coalesce with other similar cells derived from the adjacent cavities (Fig. 2d). Lytic spaces of moderate size develop at the interface with the matrix.

Attack on the cortex may also occur by radial penetration. Fig. 2f shows a wider boring hypha that, after penetrating the cortex for a certain distance, has produced side branches invading this tissue longitudinally (Fig. 2f, g). Branches developed from different types of radial penetration may merge, and tunnel

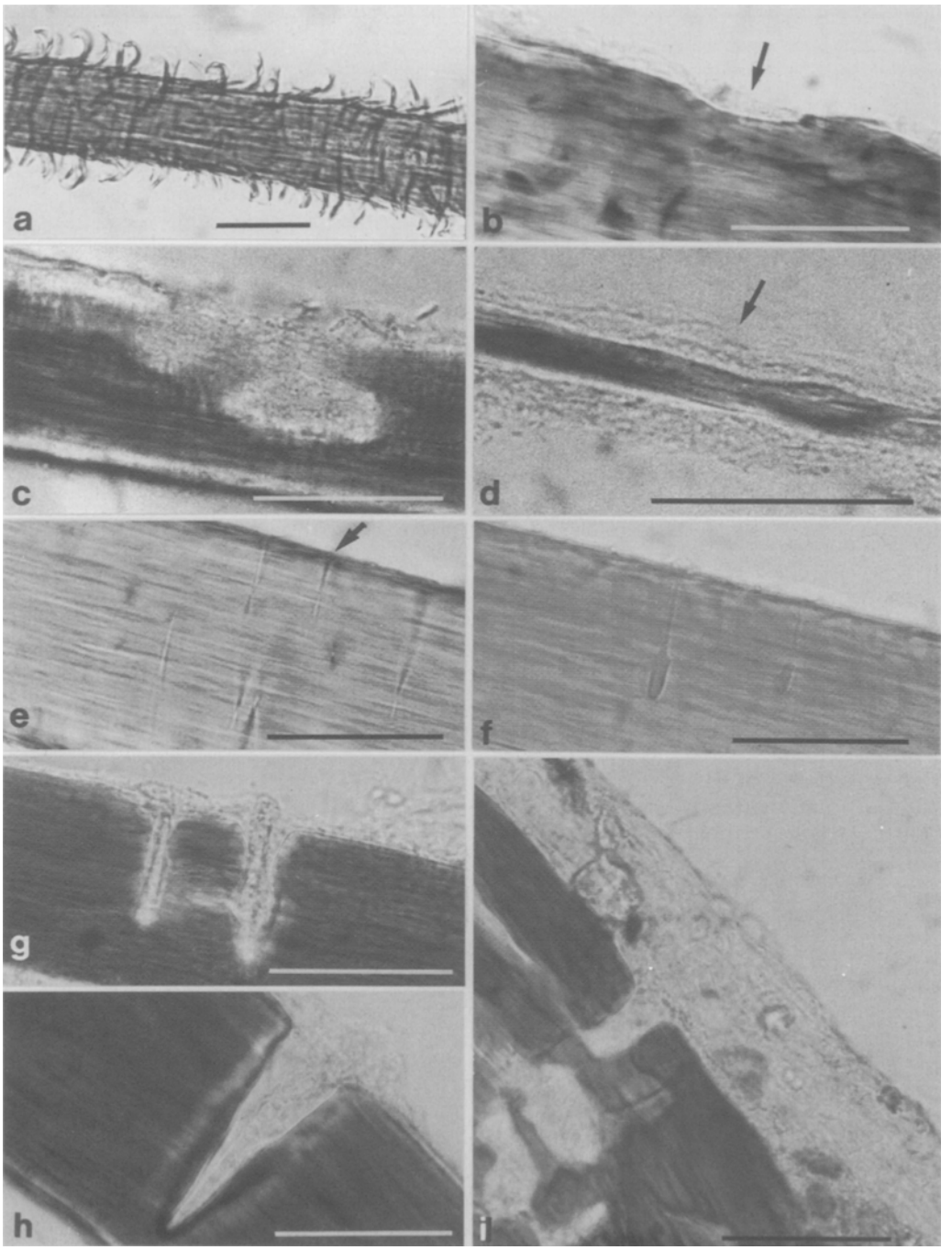

Fig. 1. Light microscopy of entire fungus-hair units. Lactophenol cottonblue. (a) *Gymnoascus reesii*: lifting of the cuticle. (b) *Myceliophthora vellerea*: surface erosion in pockets (arrow) seen in profile. (c) *Chrysosporium* an. *Pectinotrichum llanense*: surface erosion in pockets, face view. (d) *Chrysosporium* an. *Pectinotrichum llanense*: uniform surface erosion and hyphal mantel (arrow) around the hair, in advanced digestion. (e) *Beauveria bassiana*: boring hyphae (arrow). (f) *Chrysosporium* an. *Arthroderma cuniculi*: swollen boring hyphae. (g) *Trichophyton terrestre*: wider boring hyphae. (h) *Trichophyton terrestre*: perforating organ. (i) *Chrysosporium keratinophilum*: advanced digestion, with multiple confluence of lytic areas. Bars: a = 100 μm, b–i = 50 μm.

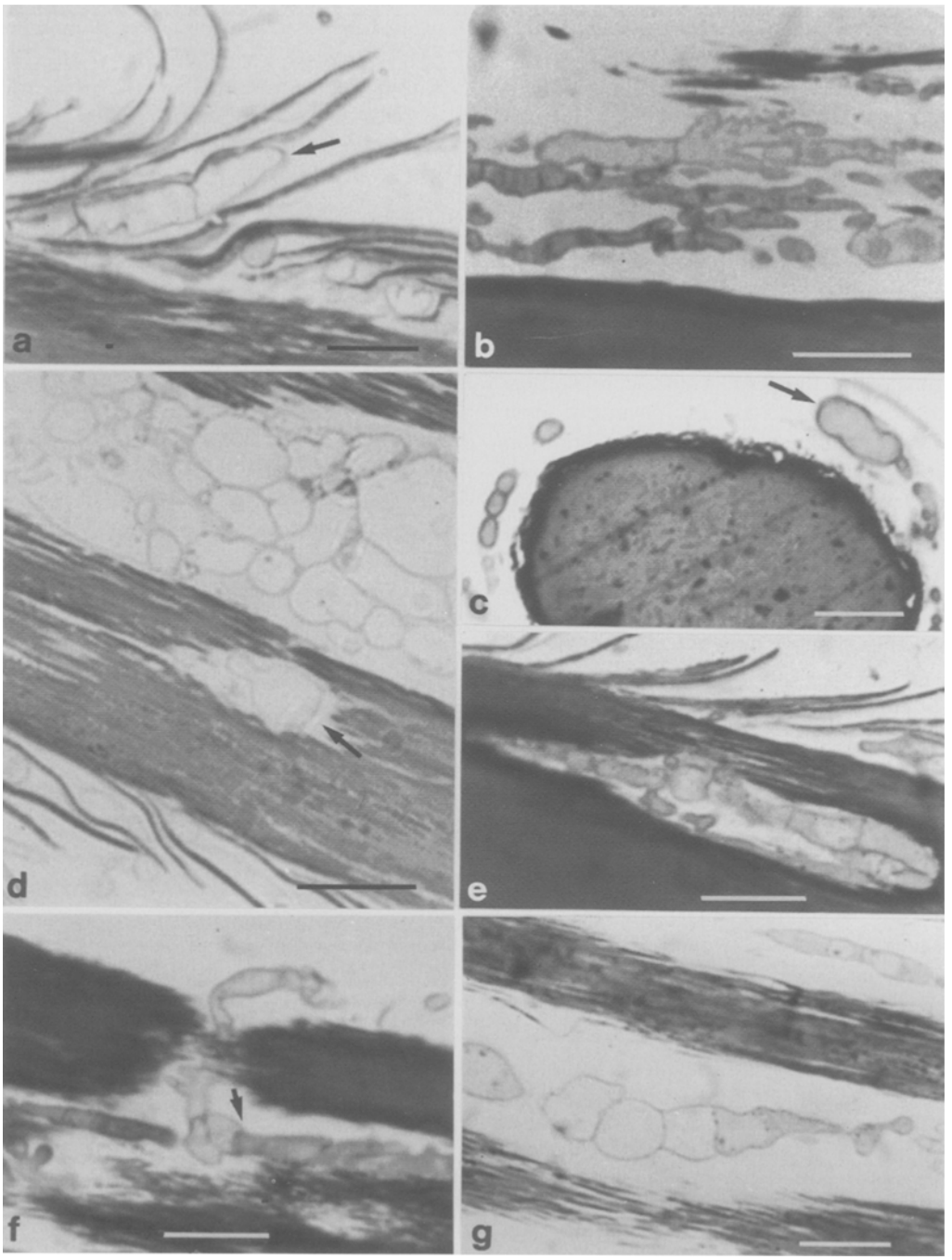

Fig. 2. Light microscopy of semithin sections of fungus-hair units. Toluidine blue. (a) *Chrysosporium* an. *Renispora flavissima*: normal hyphae and "palm of the hand" formation (arrow), from below the cuticular scales (longitudinal section). (b) *Chrysosporium* an. *Renispora flavissima*: remaining eroding mycelium after digestion (longitudinal section). (c) *Chrysosporium* an. *Pectinotrichum llanense*: transverse section of a hair and of the hyphae (arrow) involved in uniform surface digestion. (d) *Chrysosporium* an. *Arthroderma cuniculi*: hyphal structures involved, in initial (arrow) and advanced stages, in formation of an erosion pocket (longitudinal section). (e) *Chrysosporium indicum*: erosion pocket produced by growth, along the hair axis, of an initial cell. (f) *Malbranchea* st. *Uncinocarpus reesii*: wider boring hypha producing side branches (arrow). (g) *Malbranchea* st. *Uncinocarpus reesii*: a wide lytic space and evidence of advanced enzymatic attack surrounding a hypha with large cells, probably branches of radial penetration bodies. Bar= 10 μm.

within the matrix. Conspicuous lytic spaces 3–4 μm in thickness develop all around these structures; they enlarge further as digestion of the hair proceeds (Fig. 2g). Signs of enzymatic attack are visible, with separation of cells and of keratin macrofibrils.

In general, the PAS reaction gave results inferior to those of toluidine blue because it stained fungal structures less well but stained the hair more uniformly.

Scanning electron microscopy on entire, split and sectioned fungus-hair units. With this technique we obtained more detailed information on relationships between fungal structures and cuticular or cortical surfaces of the hair. We could also document some steps in the demolition sequence and the formation of lytic spaces. As in previous section on sectioned hair, figures have to be considered mere examples of a general pattern. Fig. 3a shows a three dimensional perspective of cuticle lifting, and Fig. 3b shows part of a 'palm of the hand' structure, adhering to the cortex and covered by several layers of cuticle scales, already partially digested. Fig. 3c shows a normal hypha penetrating beneath a cuticle scale, and the probable origin of a boring hypha from the end of a slightly swollen surface hypha in the form of an appressorium [4]. Fig. 3d, of a longitudinally sectioned hair, shows, in cross section, a wider boring hypha and the related lytic space. Fig. 3e shows the cortex, with cuticle already digested, with an erosion pocket occupied by a complex hyphal body whose cellular structure cannot be identified. Around it there is a small lytic space about 1 μm thick. In the surrounding zone, the cortex is beginning to break up, causing separation of cells and macrofibrillar bundles. A tunnel, free of mycelium, parallel to the hair axis and close to the cuticle, is seen in Fig. 3f. It is probably the extension of an erosion pocket and could be similar to that seen in Fig. 2e, at top left. Finally, hyphal complexes involved in uniform surface erosion of the cortex are seen in Fig. 3g, which also shows loosening of the structure of the hair involved.

Discussion

There were 19 keratinolytically active species, with seven in the genus *Chrysosporium* (*C. indicum, C. keratinophilum, C. pannicola, C. tropicum, C.* an. *Arthroderma cuniculi, C.* an. *Pectinotrichum llanense, C.* an. *Renispora flavissima*), four in the genus *Malbranchea* (*M. arcuata, M. fulva, M. sulphurea, M.* st. *Uncinocarpus reesii*), and three in the genus *Trichophyton* (*T. mentagrophytes, T. rubrum, T. terrestre*). In addition there were *Aphanoascus fulvescens, Beauveria bassiana, Geomyces pannorum* v. *pannorum, Gymnoascus umbrinus* and *Myceliophthora vellerea.* Most of these species were capable of developing structures related to surface erosion and radial penetration contemporaneously. However *Gymnoascus umbrinus, Myceliophthora vellerea,* an isolate of *C. indicum, C. tropicum* and *Trichophyton mentagrophytes* demonstrated only surface erosion. The most active keratinolytically, in terms of intensity and rapidity were *Aphanoascus fulvescens* (some isolates), *C. indicum, C. keratinophilum, C. pannicola, C. tropicum* (some isolates), *C.* an. *Pectinotrichum llanense, C.* an. *Renispora flavissima, Malbranchea* st. *Uncinocarpus reesii, Trichophyton mentagrophytes* (one isolate), *T. rubrum* and *T. terrestre* (some isolates). They were, in fact, capable of destroying about 80% of the substrate after only 3–5 days of incubation.

The model of Filipello Marchisio [1] of the morphological expression of keratinolysis, identified two basic forms of attack: surface erosion and radial penetration; many examples of these were encountered in the present work.

In addition, what we observed in semithin sections and by SEM seemed to confirm the biochemical and morphogenetic scheme proposed for *Chrysosporium tropicum* [4]. Thus, surface erosion appeared to proceed according to the sequence described by Kunert & Krajci [14] in order of increasing level of keratinization, i.e. of cystine crosslinks (cementing material, plasmalemma, endocuticle, exocuticle, layer A or, in the cortex, the matrix of the macrofibrils, microfibrils and matrix of the microfibrils). In contrast, radial penetration appeared to correspond to the ability of the fungus to concentrate secretory activity at defined points. These coincided with new growth centers of the hyphal structures which, starting from the cuticle or perhaps the cortex, penetrated the matrix perpendicularly to the hair axis. This would be true irrespective of keratinization levels in the hair components. Clearly, not all steps in the sequence could be evaluated with the techniques used in this research, and ultrastructural studies with the TEM would be useful in classifying certain aspects.

It appears that keratinolytic activity in *Beauveria bassiana, Gymnoascus umbrinus* and *Malbranchea arcuata* are here described for the first time, as

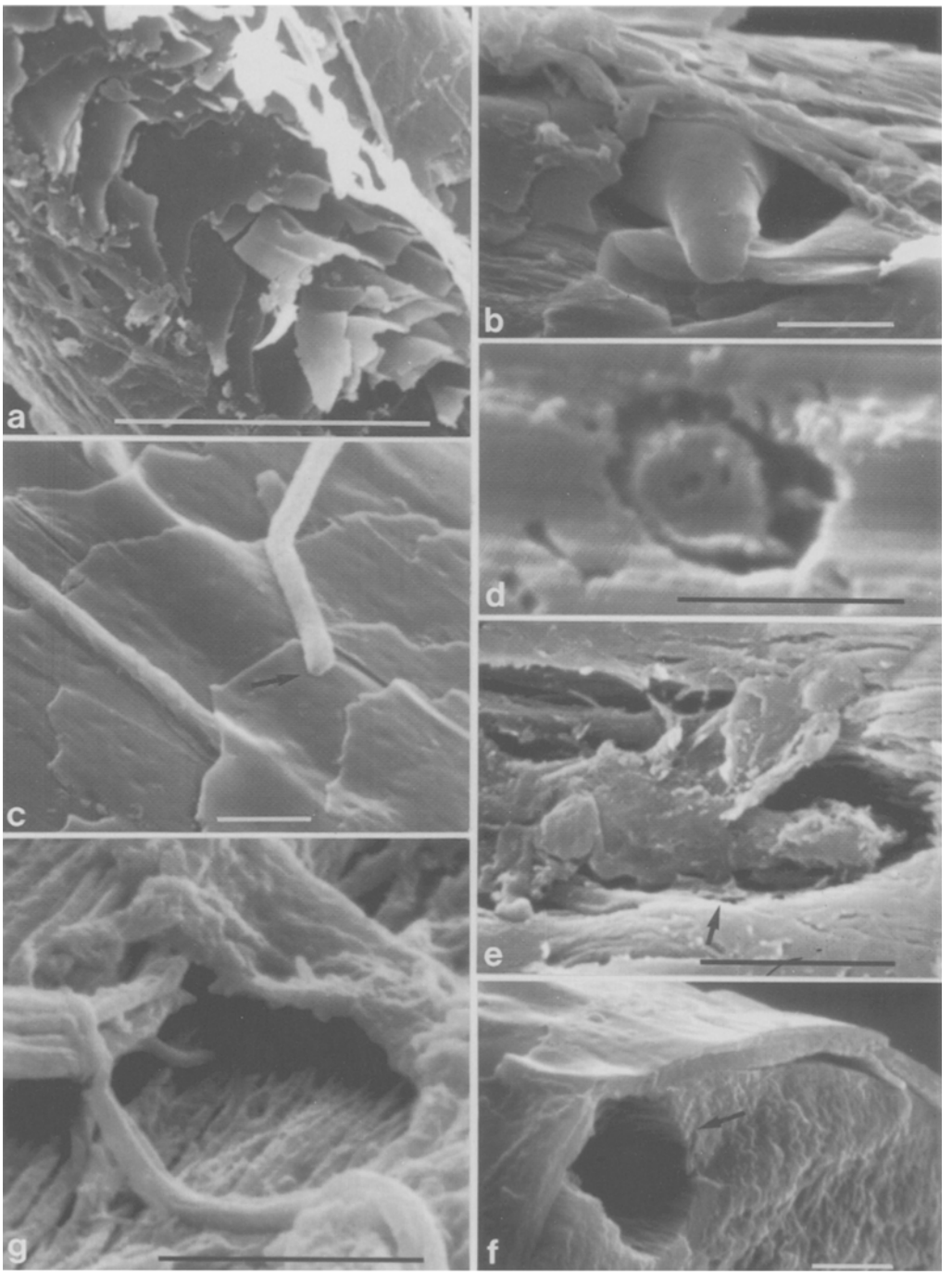

Fig. 3. Scanning electron microscopy of entire, split and sectioned fungus-hair units. (a) *Chrysosporium* an. *Arthroderma cuniculi*: lifting of the cuticle. (b) *Chrysosporium* an. *Arthroderma cuniculi*: part of a "palm of the hand" hypha attached to the cortex and covered by several layers of cuticolar scales. (c) *Chrysosporium indicum*: hypha pushing beneath a cuticular scale, and probably the start of penetration by a boring hypha (arrow). (d) *Chrysosporium keratinophilum*: cross section of a wider boring hypha in a longitudinal section of a hair. (e) *Chrysosporium indicum*: erosion pocket containing a complex hyphal body (arrow). (f) *Malbranchea* st. *Uncinocarpus reesii*: tunnel (arrow) free of mycelium, along the hair axis (split hair). (g) *Myceliophthora vellerea*: complex of hyphae involved in surface erosion of the cortex. Bars: a = 50 μm; b-f = 5 μm.

is the morphological evidence of such activity in *Chrysosporium* an. *Pectinotrichum llanense* and *C.* an. *Renispora flavissima* for which only the capacity to grow on hair substrates has been noted [18, 19]. In contrast with data in the literature [1, 19, 20], we found no keratinolytic activity associated with *Chrysosporium queenslandicum, Malbranchea gypsea, M. pulchella,* and *Ovadendron sulphureo-ochraceum.* However, such activity, like many biochemical activities of fungi, does not appear to be a constant character, or rigorously species-specific. Several authors [1, 2, 15] have already pointed this out, and we confirmed it in the present work. The results obtained with *T. mentagrophytes* and *T. rubrum* are in clear contrast with the proposal of Ajello & Georg [9] who suggested that the capacity to produce perforating organs is an important element in distinguishing the species. The results of Buchta & Heimanetk [5] and Aho [16], obtained from a large number of isolates from different sources show that indeed this character is not constant and should not be considered of diagnostic value. Nevertheless Ajello and Georg's hair penetration test specifically requires hair in dilute yeast extract broth in a Petri dish and we did not use such a substrate.

Most of the species, and especially the most active ones, are natural soil fungi [3], and secondarily make use of specific animal substrates such as skin, hairs, wool and horny matter of mammals [3, 21–29], feathers, claws and nests of birds [3, 30–36], scales of reptiles [37], insect material [18, 37] and dung of various animals [19, 37, 38]. The atmosphere could therefore be an excellent mean of transport for keratinolytic fungi, in a cycle, whose importance is difficult to evaluate, involving the soil, and the presence of man and animals.

Among the most active species we can single out those that appear best adapted to manipulation for purposes of recycling waste material and for water purification. Such fungi should preferably show no evidence of pathogenicity, either natural or experimental, and examples are *Chrysosporium pannicola, C.* an. *Arthroderma cuniculi, C. indicum, C.* an. *Pectinotrichum llanense* and *Miceliophthora vellerea.* However, before being introduced for such purposes, other parameters of pathogenicity would have to be carefully checked with these fungi, especially as our data and those in the literature suggest that in vitro tests of keratinolytic activity seem not sufficient to distinguish pathogenic from non pathogenic species. In fact, *Aphanoascus fulvescens* [3, 39], *Arthrographis cuboidea* [3], *Beauveria bassiana* [40, 41], *C. keratinophilum* [3, 18, 42], *C. tropicum* [3, 18, 42], *Geomyces pannorum* v. *pannorum* [18], *G. pannorum* v. *asperulatus* [3], *Malbranchea chrysosporoidea* [3], *M. gypsea* [3], *M. pulchella* [3], *M.* st. *Uncinocarpus reesii* [3], *Myxotrichum deflexum* [3], *Trichophyton mentagrophytes* [3, 43–46], *T. rubrum* [3, 44–46] and *T. terrestre* [3, 44] have been described as natural or experimental pathogens, but they are either not keratinolytic (*Arthrographis cuboidea, Malbranchea chrysosporoidea, Myxotrichum deflexum*) or, as far as we can see, do not show different keratinolytic behaviour from other species not known to be pathogens. There is, of course, in addition the well noted intraspecific variability of this character. Thus, although the ability to utilize keratin is in most cases associated with ability to invade and parasitize cornified tissue [40, 47], a number of other factors are probably required for initiation and development of infection [48].

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