# *Trichoderma aureoviride*: phylogenetic position and characterization

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The identity of strains identified as *Trichoderma aureoviride*/*Hypocrea aureoviridis* was reconsidered. *Trichoderma aureoviride* was isolated originally from a specimen identified as *H. aureoviridis* and thus is *H. aureoviridis*. The morphological and molecular characters of most strains identified as *T. aureoviride* differ from those of the ex-type but are more typical of *T. harzianum*, a member of sect. *Pachybasium*. Molecular data do not support inclusion of *T. aureoviride* in sect. *Trichoderma*, nor was there strong phenotypic similarity between *H. aureoviridis* and *H. rufa*. In the ITS phylogeny the *T. aureoviride* ex-type and other collections of *H. aureoviridis* form a strongly supported clade that is separate from any other recognized section of *Trichoderma*. *Hypocrea vinosa*, which was originally included in the *T. aureoviride* aggregate species concept, is distinct from *T. aureoviride*, but closely allied with *H. rufa*/*T. viride*. *Trichoderma aureoviride*/*H. aureoviridis* is a rare species, restricted to the UK and the Netherlands. We redefine *T. aureoviride*, limiting it to strains with very slow growth rate, effuse conidiation, and the ITS-1 and 2 sequence type D.

## INTRODUCTION

*Trichoderma aureoviride* (Rifai 1969) is often cited for its ability to produce enzymes (Kubicek 1982, Bruce *et al*. 1995), have biocontrol potential (Bruce *et al*. 1996, Cutler *et al*. 1999), or plant growth enhancing activities (Calvet *et al*. 1993, Camprubi *et al*. 1995). The name *T. aureoviride* was originally used for an aggregate species, but was based on single ascospore isolates from a collection identified as *Hypocrea aureoviridis* (ascomycetes, *Hypocreales*, Phillips & Plowright 1880; Rifai & Webster 1966), a species originally described from material collected on hardwood in the UK. Rifai (1969) also included the anamorph of *H. vinosa* in the *T. aureoviride* species aggregate on the basis of a perceived similarity in conidiophore branching.

The *T. aureoviride* aggr. was one of the nine aggregate species of *Trichoderma* Rifai (1969) recognized. While Rifai considered each of the aggregate species to include more than one morphologically cryptic species, Bissett (1991a) divided *Trichoderma* into five sections and included *T. aureoviride* in sect. *Trichoderma* along with the type species of the genus, *T. viride*, and other species. Gams & Bissett (1998) accepted this taxonomy. Here we examine aspects of the identity and phylogenetic placement of *H. aureoviridis*/*T. aureoviride*.

Although correct identification is essential to the communication of research results, *Trichoderma* remains taxonomically difficult. This is true, in part, because cultures present few quantifiable morphological characters that, themselves, are continuous. Additional characters useful for the taxonomy of *Trichoderma* have been obtained from DNA (see Lieckfeldt, Kuhls & Muthumeenakshi 1998a). Using DNA sequence analysis we found some species of *Trichoderma* reported to have specific biological properties were frequently misidentified when only morphology was considered (Kuhls, Lieckfeldt & Börner 1995, Lieckfeldt *et al*. 1999). Because of the frequency of misidentification of other species, we questioned whether reported strains of *T. aureoviride* also had been incorrectly identified.

We also questioned the relationship between *H. rufa* and *H. aureoviridis* implied by including their anamorphs in the same section of *Trichoderma* for three reasons. First, the teleomorph of *T. aureoviride*, *H. aureoviridis*, is anatomically and morphologically easily distinguished from *H. rufa*, the teleomorph of *T. viride* (Rifai & Webster 1966, Samuels, pers. obs.) to the extent that one would not predict a close relationship between the two. Doi (1972) included *H. rufa* and *H. aureoviridis* f. *macrospora* in different subsections of sect. *Hypocrea*. Second, there is little morphological similarity between *T. aureoviride* and *T. viride* (compare Webster 1964 with Rifai & Webster 1966, Samuels, pers. obs.; see also Samuels, Lieckfeldt & Nirenberg 1999 for a redescription of *T. viride*). Further, preliminary DNA-analysis (ITS-1- and ITS-2 sequencing)

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**Table 1.** List of strains of the *Hypocrea aureoviridis*/*Trichoderma aureoviride* complex including information about growth, grouping according to molecular data, and ITS GenBank accession number.

| Species | Strain* | Species (as reidentified) | Literature source | Geographic origin | Groups from molecular data† | ITS GenBank accession no. |
|---|---|---|---|---|---|---|
| *H. aureoviridis* (ex-type strain of *T. aureoviride*) | CBS 245.63 = SHD-M 2663 (Rifai) | *H. aureoviridis* | Rifai (1969); CBS catalogue | UK *Corylus avellana* | D | Z48819 |
| *H. aureoviridis* | CBS 525.63 | *H. aureoviridis* | Rifai & Webster, (1966); CBS catalogue | UK *Sambucus nigra* | D | AF191040 |
| *H. aureoviridis* | CBS 103.69 | *H. aureoviridis* | Bramley *1030*, CBS | UK *Acer campestre* | D | AF194004 |
| *H. aureoviridis* | IMI 138258 | *H. aureoviridis* | Bramley *1030*, CABI catalogue | UK *Acer campestre* | D | AF19021 |
| *H. aureoviridis* | CBS 138.79 | *H. aureoviridis* | CBS catalogue | NL decaying wood | D | AF194005 |
| *H. aureoviridis* | CBS 628.77 | *T.* cfr *harzianum* | van Schothorst, CBS | NL foodstuff | B | AF194006 |
| *H. aureoviridis* | IMI 355906 | *H. aureoviridis* | CABI catalogue | UK *Fagus sylvatica* | D | AF194016 |
| *H. aureoviridis* | IMI 311745 | *H. aureoviridis* | CABI catalogue | UK decorticated wood | D | AF194018 |
| *H. aureoviridis* | DAOM 172826A | *Hypocrea* sp. | Bissett (1991) | Canada rotting log, isolated from ascospores | E | AJ230662 |
| *H. aureoviridis* | DAOM 172826B | *Hypocrea* sp. | Bissett (1991) | Canada rotting log, isolated from stroma tissue | E | AJ230662 |
| *T. aureoviride* | DAOM 175924 | *T.* cfr *harzianum* | DAOM catalogue | Canada, on rotted stump of *Acer* sp. | B | AF191039 |
| *T. aureoviride* | IMI 091968 | *T. citrinoviride* | CABI catalogue | UK *Fagus sylvatica* | C | AF194017 |
| *T. aureoviride* | IMI 113135 | *T. harzianum* | produces penicillin-amidase; CABI catalogue | UK air | B | AF194019 |
| *T. aureoviride* | IMI 112086 | *T. harzianum* | CABI catalogue | Egypt soil | B | AF194020 |
| *T. aureoviride* | BBA 65638 | *T.* cfr *harzianum* | H. Nirenberg | Czech Republic cellulose producing | B | AF194007 |
| *H. vinosa* | CBS 247.63 = DAOM 167638 | *H. vinosa* | Rifai & Webster (1966) | NZ | A2 | AF191041 |
| *H. vinosa* | CBS 960.68 = DAOM 167642 | *H. vinosa* | Rifai & Webster (1966) | USA, OH sand | B | AF1910038 |
| *T. harzianum*‡ | NR 5546 | *T. harzianum* | Fujimori & Okuda (1994) | Japan, Kanagawa soil | B | AF194008 |
| *T. harzianum*‡ | NR 5555 | *T. harzianum* | Fujimori & Okuda (1994) | Japan, Tokyo soil | B | AF194009 |

**Table 1.** (*cont.*)

| Species | Strain* | Species (as reidentified) | Literature source | Geographic origin | Groups from molecular data† | ITS GenBank accession no. |
|---|---|---|---|---|---|---|
| *T. aureoviride* | NR 6883 | *T. harzianum* | Fujimori & Okuda (1994) | Japan, Kanagawa soil | B | AF194010 |
| *T. harzianum*‡ | NR 6929 | *T. harzianum* | Fujimori & Okuda (1994) | Japan, Chiba soil | B | AF194011 |
| *T. aureoviride* | NR 6931 | *T.* cfr *inhamatum* | Fujimori & Okuda (1994) | Japan, Chiba soil | B | AF194012 |
| *T. aureoviride* | NR 6938 | *T. harzianum* | Fujimori & Okuda (1994) | Japan, Okinawa soil | B | AF194013 |
| *T. aureoviride* | NR 6940 | *T. harzianum* | Fujimori & Okuda (1994) | Japan, Okinawa soil | B | AF194014 |
| *T. aureoviride* | NR 6950 | *T. harzianum* | Fujimori & Okuda (1994) | Japan, Okinawa soil | B | AF194015 |

* ATCC, American Type Culture Collection, Manasas, VA; BBA, Biologische Bundesanstalt, Berlin; CBS, Centraalbureau voor Schimmelcultures, Utrecht; DAOM, Agriculture & Agri-Food Canada Research Branch, Eastern Cereal & Oilseed Research Centre, Ottawa; IMI, CABI Bioscience, Egham, UK; NR, Nippon Roche.

† Molecular data considered: ITS-1 + ITS-2 sequences, RFLP patterns of 28S rDNA with *Msp*I and *Hha*I, and RAPD patterns produced by using primers $(GTG)_5$, $(GACA)_4$, M13 and ROB; Groupings A1, A2, B and C refer to Kindermann *et al.* (1998); D introduced here stands for 'true' *H. aureoviridis*.

‡ Strains reported as *T. aureoviride* in Fujimori & Okuda (1994), but when provided reidentified as *T. harzianum*.

revealed that the ex-type culture of *T. aureoviride* was strikingly different from ex-type cultures of all other *Trichoderma* species (Kuhls *et al.* 1997, Kindermann *et al.* 1998).

Here we discuss results from RFLP analysis of the 28S rRNA gene sequencing of the ITS-1–ITS-2 rDNA spacer region, and RAPD analysis of most available isolates identified as *H. aureoviridis* or *T. aureoviride* and *H. vinosa*. The results show that the morphological and molecular characters of the majority of the *T. aureoviride* strains are not in accordance with those of the ex-type strain. Molecular and morphological characters also readily distinguish between *H. aureoviridis* and *T. aureoviride* and show them not to be closely related. Finally we present phenotypic characters that aid in identification of *T. aureoviride*.

## MATERIALS AND METHODS

### *Fungal cultures*

Cultures identified as *Hypocrea aureoviridis*/*Trichoderma aureoviride* and *H. vinosa* were obtained from researchers and from public culture collections (Table 1). They were cultured and grown for DNA isolation as described by Kuhls *et al.* (1995).

### *DNA procedures*

DNA isolation, PCR amplification of rDNA fragments, PCR fingerprinting, RFLP analysis of 28S rDNA, and DNA sequencing of internal transcribed spacer regions from the PCR amplified rDNA fragments were performed as described by Lieckfeldt *et al.* (1998b) and Kindermann *et al.* (1998).

### *Phylogenetic analysis*

Sequences were automatically aligned using the program CLUSTAL W, and finally optimized visually. The aligned data set is deposited in TreeBase (SN536). The actual sequences have been deposited at GenBank (Table 1). Sequences for the two species of the outgroup taxon, *Hypomyces*, were from GenBank (Y17088, Y17093). Phylogenetic trees were constructed using the parsimony options of PAUP, version 4.0 beta (Swofford 1998). The most parsimonious trees were generated with the heuristic search algorithm and the following conditions: TBR branch swapping, closest sequence addition, branch collapsing option. Single gaps were treated as a fifth base. Gaps of more than three bp were handled as missing data, and an additional set of characters was added to the data matrix to signify the presence or absence of particular gaps which convey important phylogenetic information that would have been lost otherwise. The additional set of characters was defined by using a 1/0-matrix with 1 = gap and 0 = nucleotides. The matrix contains 10 1/0-characters which refer to the following parts of the 701 nt long sequence of the ITS-1–5.8S–ITS-2 sequence considered in the analysis. ITS-1: nt 113–118 (matrix character 1), 166–170 (2), 191–203 (3), 204–210 (4), 246–250 (5), 256–261 (6), 261–270 (7), 270–276 (8). ITS-2: nt 502–511 (9), 512–517 (10). Bootstrap values were calculated from 1000 replications.

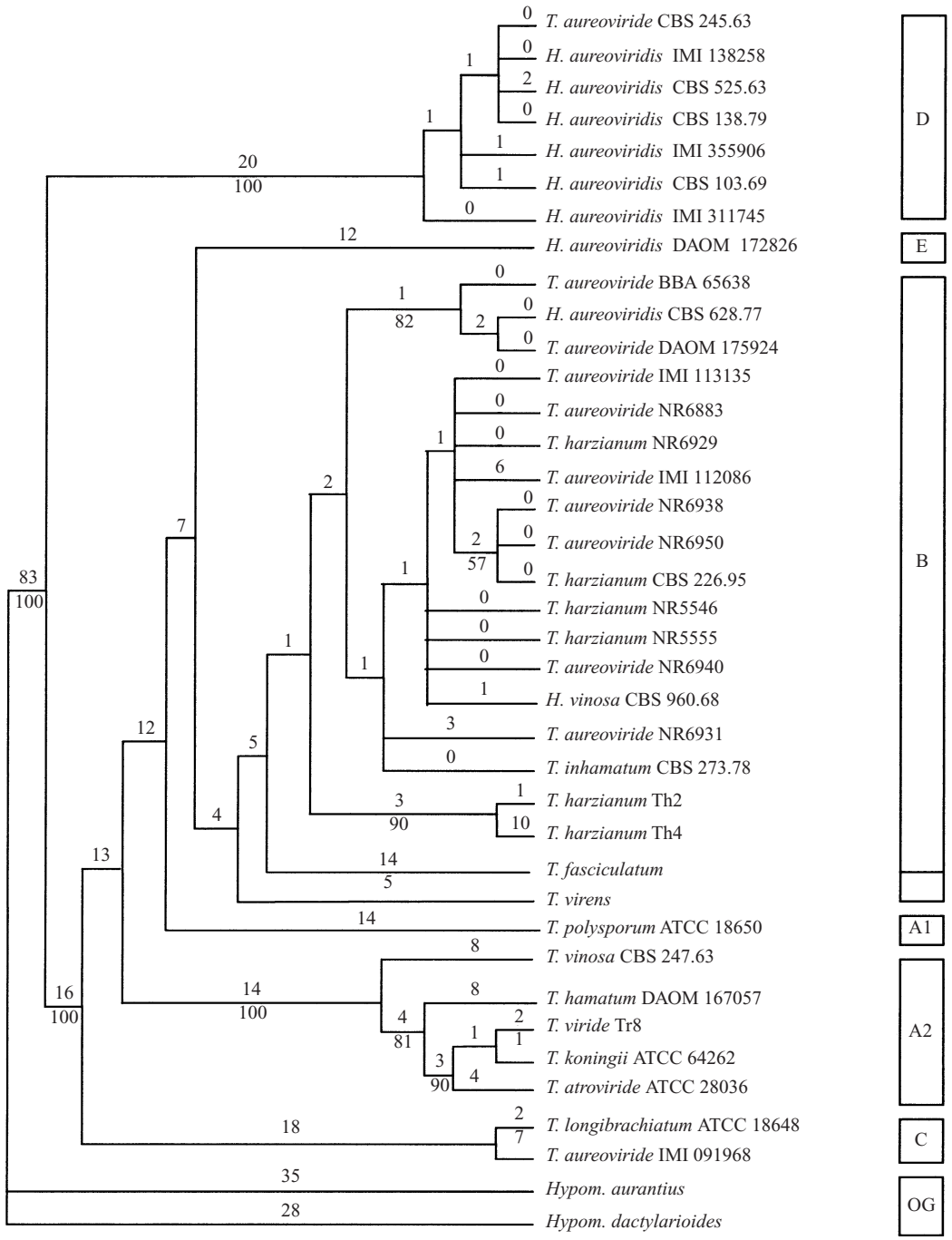



**Fig. 1.** Phylogenetic position of *Trichoderma aureoviride*/*Hypocrea aureoviridis* in *Trichoderma*. The cladogram is one of the two most parsimonious trees obtained by analysis of sequence data from the total ITS-1–5. 8S-ITS-2 rDNA region. *Hypomyces aurantius* and *H. dactyarioides* were used as outgroup. Trees are the results of a PAUP 4.02b analysis using the heuristic search option (381 steps, CI = 0.743, HI = 0257, RI = 0.869). The bootstrap support from 1000 replications is indicated below the branches; branches without values occurred in less than 50% of the bootstrapped trees. Numbers above the branches give the nucleotide differences. A1, A2, B, and C refer to main clades in *Trichoderma* ITS phylogeny as defined by Kindermann *et al.* (1998); letters D and E describe new clades for 'true' *T. aureoviride* and *Hypocrea* sp. (DAOM 172826), respectively.

### *Morphology*

Colony characters and the characteristics of conidiophores and conidia were taken from cultures grown on cornmeal dextrose agar (CMD: Difco cornmeal agar + 2% glucose), malt extract agar (MEA, Difco), potato dextrose agar (PDA, Difco), and special nutrient agar (SNA, Nirenberg 1976). The colour standard was Kornerup & Wanscher (1978). To obtain growth rates and colony characters, cultures were first inoculated onto freshly prepared CMD in Petri dishes. When the colonies were visibly growing at 20–21 °C, within 1 wk, a 5 mm diam plug of the culture was taken from the actively

growing edge and inoculated onto a freshly made 9 cm diam Petri dish containing 20 ml PDA or MEA. Morphological characters of conidiophores and conidia were obtained from cultures grown on MEA or CMD at 20 °, alternating 12 h dark and 12 h cool white fluorescent light. Where possible, 30 individuals of each morphological parameter were measured. All conidiophores and conidia were first hydrated in 3 % KOH and the measurements immediately made directly from KOH; as the KOH evaporated it was replaced by water from which additional measurements and all images were taken. There was no apparent difference between measurements made in KOH and in water.

Specimens of the *H. aureoviridis* collections from which some of the cultures were derived were obtained from the Centraalbureau voor Schimmelcultures (CBS), The University of Sheffield (SHD), and CABI Bioscience (IMI). Stromata were rehydrated in 3 % KOH. Where possible, stromata with perithecia were sectioned following the protocol of Samuels *et al*. (1998) and 30 each part ascospores and asci were measured from water.

## RESULTS

### *Molecular data*

#### *ITS sequence analysis*

The total length of the rDNA fragments amplified from the 24 strains listed in Table 1 by using the primers SR6R, 5.8SR, 5.8S, and LR1 (Vilgalys & Hester 1990) varied from 621 base pairs for *Hypocrea vinosa* (CBS 247.63) to 672 bp in *Trichoderma aureoviride* (ex-type CBS 245.63). Length differences of ITS in *Trichoderma* species belonging to different sections of the genus, a result of considerable indels in the ITS-1, were described earlier (Kuhls *et al*. 1997). With its 230 bp, the ITS-1 of the *T. aureoviride* ex-type strain (CBS 245.63) is longer than that of any other *Trichoderma* isolates examined. In contrast, lengths of ITS-2 varied only slightly (168–182 bp). The calculated G + C content of ITS-1 ranged from 54 to 60 % in all species, except for the *T. aureoviride* ex-type (65 %). The G + C content of ITS-2 was higher than that of ITS-1 (about 65 %), and displayed slightly higher values (68 %) for species in sect. *Longibrachiatum* and for the ex-type strain of *T. aureoviride*.

For phylogenetic analysis of sequence data, a 692-bp fragment (including gaps from alignment) comprising total ITS-1, 5.8 S rDNA and ITS-2 sequences of 38 taxa, including the 25 putative *T. aureoviride* and *H. vinosa* isolates, was used. Parsimony analysis was performed, using *Hypomyces aurantius* and *H. dactylarioides* as outgroup taxa, and the two most parsimonious trees were obtained. The *T. aureoviride* ex-type strain and six other *H. aureoviridis* isolates form a basal clade to all other species of *Trichoderma* (Fig. 1, clade D). The seven strains of clade D are only slightly variable in their ITS sequences. Surprisingly, there is a sequence difference in ITS-1 as well as in ITS-2 between two cultures of a single strain of *H. aureoviridis* (i.e. CBS 103.69 and IMI 138258). Repeated sequencing of new DNA fragments gave the same result. The respective sequence patterns of CBS 103.69 and IMI 138258 are compared to the other *H. aureoviridis* strains of that group (Table 2). All other isolates identified as *T. aureoviride* clustered within or in the vicinity of previously identified clades (Kuhls *et al*. 1996, Kindermann *et al*. 1998). One isolate (IMI 091968) clustered with the *T. longibrachiatum* ex-type culture (ATCC 18648) and exhibited ITS1 and two sequences identical to *T. citrinoviride*. The eight *T. aureoviride* strains of Fujimori & Okuda (1994) formed a clade together with *T. harzianum* and *T. inhamatum* (in the main clade B as described in Kindermann *et al*. 1998). No evidence for a close relationship of *T. aureoviride* to *H. vinosa* was obtained, as one isolate of the latter species (CBS 247.63) clustered with species from sect. *Trichoderma*, whereas the other (CBS 960.68) clustered in the clade containing *T. harzianum*/*T. inhamatum*. The remaining strains identified as *T. aureoviride* yielded unique sequence characteristics and potentially constitute new species. Three of them, DAOM 175924, BBA 65638 and *H. aureoviridis* CBS 628.77, exhibited only two bp difference to each other, and formed a well-supported cluster with a basal position to the *T. harzianum*/*T. inhamatum* clade. Another isolate, DAOM 172826 (A/B), occupied a position basal to clade B; exhibiting a sequence significantly different from all other strains investigated. It is defined as clade E here. The clades containing *T. polysporum* and the species from sect. *Trichoderma* (previously termed A1 and A2; Kindermann *et al*. 1998), and another clade representing sect. *Longibrachiatum* (C in Fig. 1), are well resolved in the tree.

**Table 2.** Sequence variation in ITS-1 and ITS-2 in the *Trichoderma aureoviride* ex-type strain and related *Hypocrea aureoviridis* strains. Base pairs indicate positions based on the whole 689 bp fragment which was used for the PAUP analysis.

| Species/strain | ITS-1 (bp position 185–194) | ITS-2 (bp position 434) |
|---|---|---|
| *T. aureoviride* CBS 245.63 | C C T T T T C C C C | T |
| *H. aureoviridis* CBS 525.63 | C C T T T T C C C C | T |
| *H. aureoviridis* IMI 138258 | C C T T T T C C C C | T |
| *H. aureoviridis* CBS 138.79 | C C T T T T T C C C | T |
| *H. aureoviridis* CBS 103.69 (= IMI 138258) | C C C T T T T C C C | C |
| *H. aureoviridis* IMI 355906 | C C C T T T T C C C | T |
| *H. aureoviridis* IMI 311745 | C C C T T T T C C C | T |

### *RFLP analysis of 28S rDNA*

Strains identified above as corresponding to the *T. aureoviride* ex-type strain were also distinguished by a clearly different RFLP pattern with the restriction enzymes *Hha*I and *Msp*I. Furthermore, overall 28S-rDNA RFLP patterns were consistent with the grouping established by ITS1- and ITS-2-based parsimony analysis (data not shown)

### *PCR fingerprinting*

PCR fingerprinting was performed with the telomere primers $(GACA)_4$ and $(GTG)_5$, the bacteriophage M13 core sequence, and the decamer primer ROB (Lieckfeldt *et al*. 1998a). All four primers yielded highly similar fragment patterns with six strains of the *T. aureoviride* ex-type clade, which were clearly

different from the other strains (Fig. 2A–D, lanes 1–6). However, the patterns for the strains CBS 103.69 and IMI 138258 were not identical (Fig. 2A, C–D, lanes 6 and 7). Six of the eight Fujimori & Okuda (NR) isolates also displayed very similar patterns (Fig. 2A–D, lanes 13–20), but despite their high ITS1 and 2 sequence homology with strains described as *T. harzianum*, these showed no similarity to the RAPD patterns of *T. harzianum* CBS 819.68 (Fig. 2A–D, lane 23). Two DNAs, one isolated from a single ascospore culture and the other from stroma tissue of a specimen deposited as *H. aureoviridis* (DAOM 172826), were similar to each other with minor differences in $(GTG)_5$ and M13 patterns (Fig. 2A–D, lanes 11–12). All remaining strains yielded unique patterns, regardless of the primer used.

### *Morphological investigations*

*Ascospore isolates and redescription of* T. aureoviride

Of the 20 cultures, 9 were from specimens identified as *H. aureoviridis*, of which only eight are from separate isolations: IMI 138258 is the same as CBS 103.69, and DAOM 172826 A and B are derived from a single specimen as was noted above. The ex-type culture of *T. aureoviride* (CBS 245.63) was derived from ascospores of SHD-M 2663, *H. aureoviridis*. All other strains were isolated directly from diverse substrata. The *Hypocrea* specimens from which six of these cultures were derived agreed well in morphology with the lectotype specimen of *H. aureoviridis* (K (M)!) and with the description of Rifai & Webster (1966) for the species. The anamorphs formed in these cultures agreed well with Rifai & Webster (1966). These six specimens and their cultures are taken to be 'true' *H. aureoviridis*, and the description given below is based on them.

Although some strains grew faster than others (most notably CBS 138.79), all grew slowly relative to other *Trichoderma* isolates, reaching only 40–75 mm diam after one wk on MEA and PDA at the optimum temperature of 20 °. On average, colonies incubated in alternating light and darkness grew slightly slower than those incubated in darkness with only intermittent and infrequent exposure to light. After 64 h on PDA and SNA there was no growth at 30 ° or 35 °. On MEA at 20 ° in darkness after 1 wk colonies were uniformly flat and velvety. Aerial mycelium comprised short hyphae that formed a uniform lawn over the colony; concentric rings were generally lacking except for IMI 355906 and IMI 311745, in which a centrally located ring of intense conidial production was observed. There was more dense production of aerial mycelium on PDA.

Within one week, cultures grown on MEA under a regimen of 12 h darkness/12 h cool white fluorescent light produced more or less intense yellow pigment that was most easily seen in the colony reverse. No pigment was observed in cultures incubated in darkness with only intermittent and infrequent exposure to light. Needle-like, yellow crystals were observed in MEA cultures of IMI 355906 and IMI 311745; crystals were not observed in the ex-type strain (CBS 243.63) but yellowish, amorphous material was produced sporadically in MEA cultures of it.

Conidial production equally abundant on MEA and PDA, in either darkness or in light, but poor on CMD. Conidiophores (Figs 3–4, 7) 50–100 µm long, smooth, typically branched along the length in a verticillate fashion, or branching at or near the tip and then in a gliocladium-like fashion, arising from the surface of the agar or from aerial hyphae typically formed in a continuous and uniform lawn throughout the colony with no tendency to form pustules. Phialides divergent from the point of basal attachment to the conidiophore and irregularly or regularly disposed along the conidiophore, or forming a broad penicillus toward the tip, straight, tapering slightly from base to tip, 9–15 µm long, 2.5–3 µm wide in the middle, 2.5–3 m wide at the base; periclinal thickening visible at the tip, the collarette flared; the phialides persistent with age.

Conidia typically clavate (Fig. 5) to ellipsoidal or subglobose (Fig. 6) (l/w = 1.4–1.8) often with a truncate or slightly protuberant base, 3.5–5 × 2.5–3 µm, smooth; held in drops of clear, virtually colourless (extremely pale green when seen with the stereo microscope) watery liquid that, in mass, typically gave the colony a greyish green color (27B5). IMI 355906 and IMI 311745 differed from the other 4 strains in producing zonate colonies. The zonation in conidial production was especially marked on PDA cultures incubated in the light and was accompanied by the production of olive yellow to olive pigment in the colony (3D-F8).

### *Teleomorphs*

With the exception of CBS 138.79, collected in The Netherlands, all the *Hypocrea* collections were made in the UK and the cultures were derived from ascospores. The strain CBS 628.77 was identified as *H. aureoviridis*, but was isolated from foodstuff and not ascospores.

The Canadian specimen (DAOM 172826 A and B), originally identified as *H. aureoviridis*, was superficially similar in gross morphology and in green ascospores, but differed from typical *H. aureoviridis* in having larger ascospores and in an anamorph that resembles *T. strictipilis* (Bissett 1991b). Additionally, the anamorph of DAOM 172826 was easily distinguished from *T. aureoviride*. DAOM 172826 did not cluster with any other strains and resembles *H. aureoviridis* f. *macrospora* Doi (Doi 1972) both in anamorph and teleomorph. Original cultures of the form are no longer available.

### *Conidial isolates*

Fourteen strains that had been identified as *T. aureoviride* were derived directly from diverse natural substrata. All were fast growing, covering a 9 cm diam Petri dish within 4 d at 25 ° and all grew at 35 °; all had conidia that were smaller than is typical for *T. aureoviride*. Most fell into ITS group B; their conidiophores and subglobose conidia were typical of *T. harzianum* (*sensu* Gams & Meyer 1998; Figs 8–10). Several of these strains produced an intense yellow pigment on PDA and their hyphae were filled with amorphous yellow material. The single strain in ITS group C (IMI 091968) was typical of *T. citrinoviride*, a member of the *H. schweinitzii* complex (Samuels *et al*. 1998).

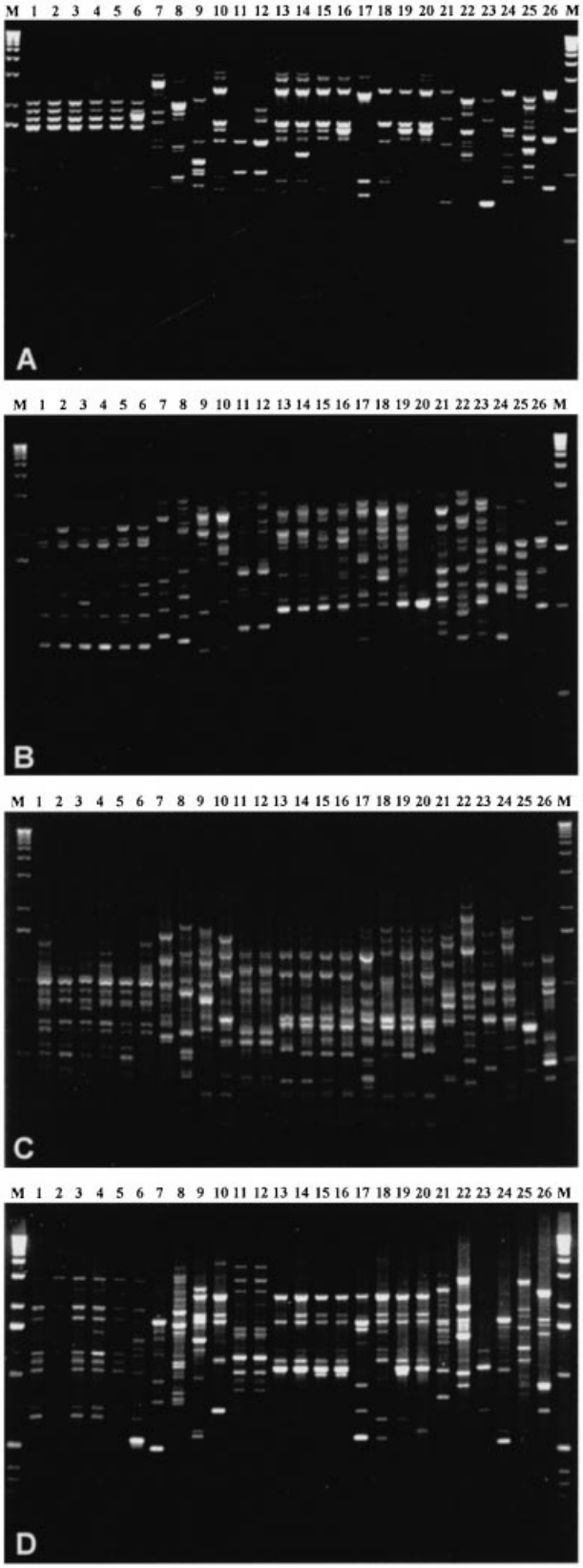


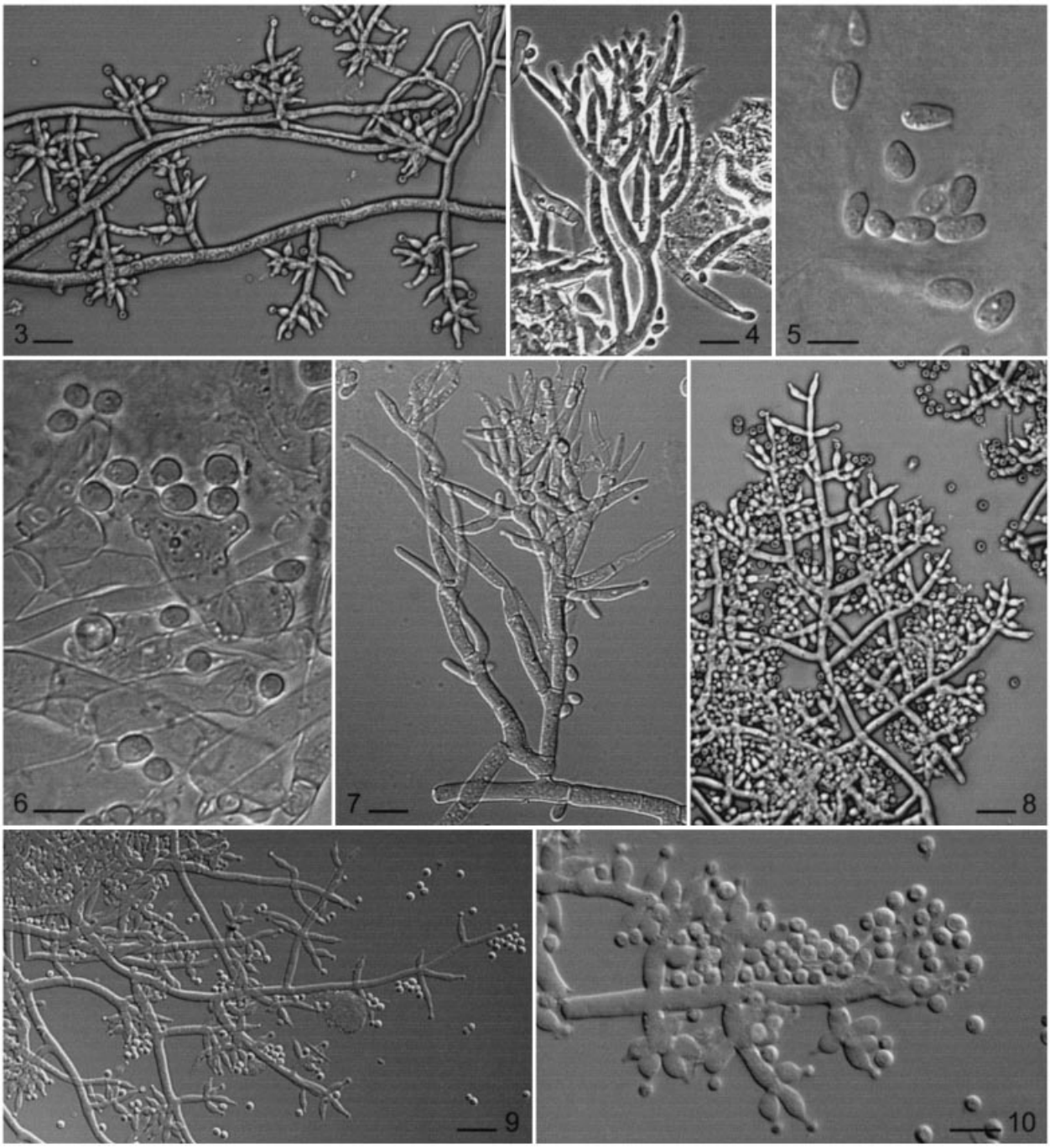

**Fig. 2.** *Hypocrea aureoviridis*/*Trichoderma aureoviride* PCR fingerprinting patterns of genomic DNA revealed by primers M13 (A), $(GTG)_5$ (B), $(GACA)_4$ (C), and ROB (D). *Lanes*: *M*, 1 *kb ladder* (*Life Science Technologies*). 1, *T. aureoviride* ex-type CBS 245.63. 2, *H. aureoviridis* CBS 138.79, 3, *H. aureoviridis* IMI 355906. 4, *H. aureoviridis* IMI 311745. 5, *H. aureoviridis* CBS 103.69. 6, *H. aureoviridis* IMI 138258. 7, *H. aureoviridis* CBS 628.77. 8, *T. aureoviride* IMI 091968. 9, *T. aureoviride* IMI 113135. 10, *T. aureoviride* IMI 112086. 11, *H. aureoviridis* DAOM 172826A. 12, *H. aureoviridis* DAOM 172826B. 13, *T. harzianum* NR 5546. 14, *T. harzianum* NR 5555. 15, *T. harzianum* NR 6883. 16, *T. harzianum* NR 6929. 17, *T. harzianum* NR 6931. 18, *T. harzianum* NR 6938. 19, *T. harzianum* NR 6940. 20, *T. harzianum* NR 6950. 21, *T. aureoviride* BBA 65638. 22, *H. vinosa* CBS 247.63. 23, *T. harzianum* CBS 819.68. 24, *T. inhamatum* CBS 273.78. 25, *T. longibrachiatum* ATCC 18648. 26, *T. koningii* ATCC 64262.

## DISCUSSION

Rifai & Webster (1966) redescribed and lectotypified *Hypocrea aureoviridis*, and were the first to link the species to a *Trichoderma* anamorph, later described as the *T. aureoviride* aggregate (Rifai 1969). This new *Trichoderma* name was based on ascospore isolations from a collection identified by Rifai & Webster (1966) as *H. aureoviridis* (SHD-M 2663 = CBS 245.63). There is no reason to doubt that Rifai & Webster correctly identified *H. aureoviridis* and that *T. aureoviride* is *H. aureoviridis*. They provided a complete description and illustrations of stromata of *H. aureoviridis*. They characterized the species as very slow growing with few aerial hyphae, a watery white mycelium, yellow needle-like crystals and a dirty yellow to brownish reverse. With the exception of the crystals, which were lacking from most of the cultures that we studied, these characteristics were noted for the ex-type strain (CBS 245.63) and the six *H. aureoviridis* strains clustering with it. Yellow needle-like crystals were found in two of the more recent IMI cultures. Despite the characteristic slow growth rate that Rifai & Webster documented for the species, which is unusual in *Trichoderma* and *Hypocrea* (compare growth rates for other species of *Trichoderma* and *Hypocrea* in Samuels *et al*. 1998, Lieckfeldt *et al*. 1998b, Seaby 1996, Samuels, pers. obs.), the species has been repeatedly misidentified. If the name of an organism is a key to communication about it, then – as has often happened in *Trichoderma* – communication about *T. aureoviride* has failed. Reports attributing activities and properties to *T. aureoviride* are erroneous, referring mainly to *T. harzianum*. In the present work we re-emphasize diagnostic characters of *T. aureoviride* to enable its correct identification while, at the same time, leading diagnosticians to other more likely names.

It is difficult to speculate as to why this species has been misidentified so frequently, but the production a diffusing yellow pigment in agar media may have been misleading. In addition to yellow crystals, Rifai (1969) attributed a diffusing brownish yellow pigment to *T. aureoviride*. Diffusing yellow pigment is found also in distantly related species of *Hypocrea*, including *H. schweinitzii* (*T. citrinoviride*) and other members of the *H. schweinitzii* complex (Samuels *et al*. 1998) as well as in some strains of *T. harzianum* (*Trichoderma* sect. *Pachybasium*; Seaby 1996, Samuels pers. obs.).

*Trichoderma aureoviride* is unusual in *Hypocrea* because of the tendency for its conidiophores to be finite in length, as opposed to typical *Trichoderma*'s where well-defined conidiophores are not formed (Samuels, 1996). Further, its phialides tend to be disposed in a gliocladium- or verticillium-like fashion (Figs 4, 7) and taper uniformly from base to tip rather




**Figs 3–10.** *Hypocrea aureoviridis*/*Trichoderma aureoviride*, conidiophores and conidia. **Figs 1–5.** CBS 245.63, the ex-type culture of *T. aureoviride*. **Fig. 6.** IMI 311745. **Fig. 7.** IMI 355906. **Figs 8–10.** *T. harzianum* misidentified as *T. aureoviride*. **Fig. 8** NR 6938. **Figs 9–10.** NR 6931. Bars: Figs 3–4, 7–9 = 10 µm; Figs 5–6, 10 = 5 µm.

than being enlarged in the middle (compare Figs 4, 7 with Figs 8, 10). Finally, while *Trichoderma* conidia are typically held in powdery masses, we know of only a few species that produce their conidia in watery heads, namely *T. virens*, *H. gelatinosa*, and *H. lutea* (‘*Gliocladium*’ *viride*). *Trichoderma virens* and *H. aureoviridis* are not obviously closely related (Fig. 1). *Hypocrea lutea* and *H. gelatinosa* are the subject of continuing study in our laboratory (P. Chaverri, unpubl.).

In the phylogenetic analysis the *H. aureoviridis* clade was very strongly supported. Although Rifai (1969) included the anamorph of *H. vinosa* in the morphological concept of *H. aureoviridis*, molecular characters do not support this placement. The anamorph of *H. aureoviridis* is easily distinguished from that of *H. vinosa* at the species level, but Kindermann *et al.* (1998) demonstrated that morphology in *Trichoderma* is not necessarily a good predictor of interspecies relationships and so it was reasonable for Rifai & Webster to propose a relationship between *H. vinosa* and *H. aureoviridis*. *Hypocrea vinosa* was described in the last century from a specimen collected in New Zealand. We are only aware of one culture to have been derived from ascospores of a specimen identified as *H. vinosa* (CBS 247.63) and that clusters in a clade that includes *H. rufa* (Fig. 1, as its anamorph *T. viride*). A second culture, isolated directly from soil in the USA and identified as

*H. vinosa*, is *T. harzianum*. Our results confirm those of Bissett (1991b) and Gams & Bissett (1998) in regarding *H. aureoviridis*/*T. aureoviride* as a phylogenetic species.

Although there was some genetic variation within the *T. aureoviride* clade, those branches were not well supported. The surprising deviation in ITS-1 and 2 sequences and in RAPD patterns of the two cultures of the single *H. aureoviridis* strain CBS 103.69 and IMI 238258 is perhaps a result of the separate cultivation/storage history of the respective cultures in different culture collections over approximately 30 years. A contamination of one of the cultures can be excluded, because contamination should have resulted in a much more pronounced genetic difference; it is highly unlikely that the contaminant would have been another strain of *H. aureoviridis*. There was no evidence for multiple ITS types in *H. aureoviridis* cultures as repeated sequencing of new DNAs gave exactly the same unique sequence for each culture. There was some phenotypic variation within the clade, for example IMI 311745 has smaller conidia and a faster growth rate, but we cannot assess the taxonomic significance of these differences given the small number of specimens available.

In an updated key to *Trichoderma*, Gams & Bissett (1998) maintained *T. aureoviride* in sect. *Trichoderma*, and described it on the basis of isolate CBS 283.79, which originated from the same location as strain CBS 138.79. The latter was included in the present study and shown to be a member of the clade containing the ex-type strain, CBS 245.63. However, our molecular data do not support the inclusion of *T. aureoviride* in sect. *Trichoderma* or in any other recognized section of the genus. In contrast, the strains described by Fujimori & Okuda (1994) as *T. aureoviride* display physiological, morphological and molecular characters that correlate with those reported for *T. harzianum*. In fact, Okuda *et al*. (1982) and Fujimori & Okuda (1994) mentioned the similarity of *T. aureoviride* to *T. harzianum*, and by the time they were obtained for this study some had been reidentified as *T. harzianum* (see Table 1).

Strain DAOM 172826, which according to Bissett (1991b) has affinity *to H. aureoviridis* and resembles *T. atroviride*, is similar to *H. aureoviridis* in phenotypic characters (stroma), but is otherwise not very much like *T. atroviride*. Molecular data place this strain basal to the former sect. *Pachybasium* (clade B in Kindermann *et al*. 1998). On the other hand, there is a short sequence motif in ITS-1 at bp 166–170 (AAAAA) in which DAOM 172826 is similar to that of the *T. aureoviride* ex-type group (AAGAA) and absent in all other *Trichoderma* and *Hypocrea* isolates investigated so far. According to our recent findings, we introduce a new group, 'E', for *Hypocrea* sp. DAOM 172826; this *Hypocrea* is possibly *H. aureoviridis* f. *macrospora* (Doi 1972), based on the description and illustrations of that taxon and our study of a paratype specimen (NY).

In summary, *H. aureoviridis* is a rare species seemingly restricted to northwestern Europe (The Netherlands and UK). It has been encountered only in isolations from ascospores. The data clearly demonstrate that many strains identified as *T. aureoviride* are actually members of sect. *Pachybasium* with a strong affinity to *T. harzianum* or *T. inhamatum*. High GC-content of the ITS1 and two sequences and the comparatively long sequence length place *H. aureoviridis* in an isolated position in *Hypocrea*. In contrast to the suggestion that it is closely related to *H. rufa*/*T. viride* (Bissett 1991a, Gams & Bissett 1998), it is quite distinct from them and possibly represents a previously unrecognized group within *Hypocrea*. The anamorph, with conidia held in watery heads on gliocladium-like conidiophores, suggests relationship to *H. gelatinosa* and *T. virens*; ITS rDNA Sequences for *H. gelatinosa* are not yet available. Although our data (Fig. 1) do not show a close relationship between *H. aureoviridis* and *T. virens*, we cannot rule out the possibility that such a relationship will be revealed with the study of additional taxa. Although the biogeographic study of *Hypocrea* is only in an early phase, we have not yet encountered a species that has such a restricted geographic distribution. The apparent restriction of *H. aureoviridis* to northwest Europe, despite extensive collecting in North America and Europe, and its slow growth rate in culture, suggest that it has adapted to particular growth factors found in its habitat. As a result of the geographic isolation there has been little gene flow and, thus, little genetic variation in the species.

On the basis of our results, we redefine *T. aureoviride*, limiting it to *Trichoderma* strains that are anamorphic *H. aureoviridis* and that have a very slow growth rate, effuse conidiation, and the ITS-1 and 2 sequence type D.

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