Hypocrea/Trichoderma (*Ascomycota*, *Hypocreales*, *Hypocreaceae*): species with green ascospores

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Abstract: The systematics of species of *Hypocrea* with green ascospores and their *Trichoderma* anamorphs is presented. Multiple phenotypic characters were analysed, including teleomorph and anamorph, as well as colony morphology and growth rates at various temperatures. In addition, phylogenetic analyses of two genes, the RNA polymerase II subunit (RPB2) and translation elongation factor 1-alpha (EF-1α), were performed. These analyses revealed that species of *Hypocrea* with green ascospores and *Trichoderma* anamorphs are derived from within *Hypocrea* but do not form a monophyletic group. Therefore, *Creopus* and *Chromocrea*, genera formerly segregated from *Hypocrea* only based on their coloured ascospores, are considered synonyms of *Hypocrea*. The present study showed that phenotypic characters alone are generally not helpful in understanding phylogenetic relationships in this group of organisms, because teleomorph characters are generally highly conserved and anamorph characters tend to be morphologically divergent within monophyletic lineages or clades. The species concept used here for *Hypocrea/Trichoderma* is based on a combination of phenotypic and genotypic characteristics. In this study 40 species of *Hypocrea/Trichoderma* having green ascospores are described and illustrated. Dichotomous keys to the species are given. The following species are treated (names in bold are new species or new combinations): *H. albocornea, H. atrogelatinosa, H. aureoviridis/T. aureoviride,* ***H. candida/T. candidum****, H. catoptron/****T. catoptron****, H. centristerilis,* ***H. ceracea/T. ceraceum****, H. ceramica/****T. ceramicum****,* ***H. chlorospora/T. chlorosporum****, H. chromosperma/****T. chromospermum****,* ***H. cinnamomea/T. cinnamomeum****,* ***H. clusiae****, H. cornea,* ***H. costaricensis****,* ***H. crassa****/T. crassum, H. cremea/****T. cremeum****, H. cuneispora/****T. cuneisporum****, H. estonica/****T. estonicum****, H. gelatinosa/****T. gelatinosum****, H. gyrosa, H. lixii/T. harzianum, H. macrospora, H. melanomagna/****T. melanomagnum****, H. nigrovirens/****T. nigrovirens****, H. phyllostachydis/****T. phyllostachydis****, H. rugulosa,* ***H. sinuosa/T. sinuosum****, H. spinulosa,* ***H. straminea/T. stramineum****, H. strictipilosa/T. strictipile, H. substipitata, H. sulawesensis, H. surrotunda/****T. surrotundum****, H. tawa/****T. tawa****,* ***H. thailandica/T. thailandicum****,* ***H. thelephoricola/T. thelephoricola****, H. tuberosa,* ***H. velenovskyi****, H. virens/T. virens,* and *H. virescentiflava*. The following species are excluded: *H. andinogelatinosa, H. dacrymycella, H. dichromospora, H. palmicola, H. pseudogelatinosa, H. subalbocornea, H. subatrogelatinosa, H. tropicosinensis, H. viscidula, H. viridis,* and *Chromocrea leucostroma*.

Key words: anamorph-teleomorph connection, monograph, phylogeny, RNA Polymerase gene, RPB2, species concept, systematics, Translation Elongation Factor 1-α gene.

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INTRODUCTION

Members of the *Hypocreales* (Fungi, *Ascomycota*) are common in all types of moist forests (for general references see Rossman *et al.* 1999). These fungi are easily recognized by their brightly coloured fructifications. The most conspicuous members, species of *Hypomyces* (Fr.) Tul. (*Hypocreaceae*), parasitize mushrooms and bracket fungi. Another commonly encountered genus is *Hypocrea* Fr. (*Hypocreaceae*). Species of *Hypocrea* and its anamorph *Trichoderma* Pers. are commonly encountered in humid tropical or subtropical forests although they also occur in arid, temperate or boreal forests, even in the most extreme north and south latitudes. The *Hypocrea* teleomorph can be found on wood, on other members of the Ascomycota, on resupinate basidiomycetes, or on perennial bracket fungi in varying stages of decay, and less commonly on herbaceous substrata. The *Hypocrea* form typically presents itself as a cushion-shaped, brightly or lightly coloured, fleshy stroma that is no more than 5 mm in diameter, although stromata of some species may be several centimeters in extent and may even be club-shaped or turbinate. All investigated *Trichoderma* species are intimately related to *Hypocrea*, and, increasingly, named *Trichoderma* species are being shown to be the anamorphs of *Hypocrea* species (Kuhls *et al.* 1996, Samuels *et al.* 1998, Chaverri *et al*. 2001a). The *Hypocrea* form is rarely seen in culture. On the other hand, it is in the *Trichoderma* state that members of this genus are recovered in ecological investigations or cultivated in connection with commercial applications. The *Trichoderma* forms are commonly isolated from soils but also sporulate on moist wood, mushrooms, or bracket fungi in forests, where they are easily recognized by their masses of conidia that are usually green, but less commonly white or yellow. They are also found in diverse habitats: for example, they may be found in water-damaged buildings, or as endophytes within the trunks of asymptomatic tropical forest trees (Evans *et al.* 2003). In addition, they may be isolated as etiologic agents of opportunistic infection in immunocompromised humans (Kuhls *et al.* 1999). The fact that many *Trichoderma* species have demonstrated antifungal or plant-growth-stimulating activities has led to their exploitation as biological control agents, and in this connection some isolates are used in commercially available applications.

More than 200 species of *Hypocrea* have been described, yet the genus has never been monographed. Recent systematic research suggests that it is not possible to identify a *Hypocrea* species unless its *Trichoderma* anamorph is known (Samuels *et al.* 1998, Chaverri *et al.* 2003a, Lu *et al.* 2003). Although only about 50 species of *Trichoderma* have been described, exploration of new geographical locations and ecological niches has revealed many additional undescribed species. Because the anamorph is the form most commonly connected to information on the economic and ecological significance of these organisms, it is essential to clarify the biology of *Trichoderma* and *Hypocrea* in a unitary way. Holomorphs must be studied in order to effectively determine both life cycles and species concepts.

The genus *Hypocrea* (anamorph *Trichoderma*)

Hypocrea was first described by Elias Fries in 1825, based on *Sphaeria rufa* Pers. : Fr., a species with hyaline ascospores. Currently, the type species of the genus is represented by *Hypocrea rufa* (Pers. : Fr.) Fr. *Hypocrea* is generally characterized by having perithecia embedded in fleshy stromata formed by pseudoparenchymatous tissue or highly compacted hyphae. *Hypocrea* has eight 1-septate ascospores that disarticulate at the septum early in their development, producing 16 part-ascospores in each ascus. Even though the disarticulation of ascospores is one of the distinguishing characters of the genus, other genera such as *Aphysiostroma*, *Arachnocrea*, *Dialhypocrea*, *Podostroma*, *Protocrea*, *Pseudohypocrea*, and *Sporophagomyces* also have disarticulating ascospores (Rossman *et al.* 1999). Recent studies using DNA sequence data suggest that *Podostroma* (H.L. Chamberlain, pers. comm.) and *Protocrea* (B.E. Overton, pers. comm.) are congeneric with *Hypocrea*. *Podostroma* was defined to include species with upright, stipitate stromata. In several studies, similarities were noted between *Podostroma* and *Hypocrea*, which differ only in the stalked stroma of the former (Boedijn 1934, Doi 1966, Rossman *et al.* 1999). Other characteristics of *Podostroma*, such as morphology of the stromatal tissue, perithecia, and anamorphs and ecology, are indistinguishable from corresponding features of *Hypocrea*. *Aphysiostroma, Arachnocrea, Dialhypocrea, Pseudohypocrea*, and *Sporophagomyces*, which are similar to *Hypocrea* in the disarticulation of their ascospores, have distinct teleomorph and anamorph morphology. Most notably, ascospores of *Arachnocrea, Pseudohypocrea,* and *Sporophagomyces* are biconical. *Arachnocrea* and *Sporophagomyces* are mycoparasitic. Recent molecular-phylogenetic studies show that *Aphysiostroma* and *Arachnocrea* are basal to *Hypocrea s. str.* (Põldmaa *et al.* 1999, Zhang & Blackwell 2002). *Hypocrea pallida* Ellis & Everh., a parasite of members of the *Aphyllophorales sensu lato*, was placed in *Hypocrea* because of its disarticulating, 2-celled ascospores and perithecia embedded in a pseudostroma or subiculum. However, *H. pallida* has a gliocladium-like anamorph that is similar to *G. penicillioides* Corda, the type species of the genus *Gliocladium* and the anamorph of *Sphaerostilbella aureonitens* (Tul.) Seifert *et al.*

Table 1. Species published in *Chromocrea* and *Creopus.*

***Hypocrea* Fr.**	***Creopus* Link**	***Chromocrea* Seaver**
H. aureoviridis Plowr. & Cooke 1880		*Ch. aureoviridis* (Plowr. & Cooke) Petch 1938
H. ceramica Ellis & Everh. 1892		*Ch. ceramica* (Ellis & Everh.) Seaver 1910
H. cupularis (Fr.) Sacc. 1883		*Ch. cupularis* (Fr.) Petch 1938
H. gelatinosa (Tode : Fr.)Fr. 1849	*Cr. gelatinosus* (Tode : Fr.) Link 1833	*Ch. gelatinosa* (Tode:Fr.) Seaver 1910
		Ch. marathwadi Tilak & Gaikwad 1978
H. nigricans (Imai) Yoshim. Doi 1972		*Ch. nigricans* Imai 1935
H. palmicola Berk. & Cooke 1873	*Cr. palmicola* (Berk. & Cooke) Boedjin 1951	
H. spinulosa Fuckel 1869	*Cr. spinulosus* (Fuckel) Moravec 1956	*Ch. spinulosa* (Fuckel) Petch 1950
		Ch. substipitata Seaver 1910
	Cr. velenovskyi Z. Moravec 1956	

Molecular data show that *H. pallida* is more closely related to *Hypomyces* and *Sphaerostilbella* than to *Hypocrea* (Põldmaa *et al.* 1999).

Ascospore pigmentation has been used to characterize genera in the *Hypocreales*. Two genera, *Creopus* Link and *Chromocrea* Seaver, were segregated from *Hypocrea* because of their green ascospores. In 1791, Tode described *Sphaeria gelatinosa* Tode, a green-spored species that was placed in *Hypocrea* by Fries (1849) as *H. gelatinosa* (Tode : Fr.) Fr. Link (1833) based his description of the genus *Creopus* on *S. gelatinosa*. Even though Link did not explicitly state that *Creopus* was segregated based on green ascospores, most of the species subsequently placed in the genus have been those possessing this feature. A total of four species have been included in *Creopus* (Table 1). Later, Seaver (1910) proposed *Chromocrea* for species of *Hypocrea* with green ascospores; he designated *C. gelatinosa* as the type, thus making *Chromocrea* a later, homotypic synonym of *Creopus.* Eight species have been included in *Chromocrea* (see Table 1). *Sarawakus* Lloyd is the only other hypocrea-like genus that has green ascospores. In the *Hypocreales*, apart from *Hypocrea*, only members of the genus *Viridispora* Samuels & Rossman (*Nectriaceae*) and some soon-to-be-published species of *Neonectria* (Samuels pers. comm.) have green ascospores.

The first 'modern' treatment of *Hypocrea* was published by Dingley (1952, 1957). She described the anatomy of the stroma and also undertook cultural studies to link species to their anamorphs. Several new species of *Hypocrea* and their anamorphs were described from New Zealand (Dingley 1952, 1957). Dingley identified all of the *Hypocrea* anamorphs as being typical of *T. viride* Pers. : Fr., despite differences among them that were clearly visible in her illustrations. Developing on the works of Webster (1964), Webster & Rifai (1968) and Rifai & Webster (1966a, b), Rifai (1969) published a critical re-examination of *Trichoderma* in which he included nine aggregate species. Since that time, Doi has produced a floristic monograph of *Hypocrea* and its relatives in Japan (Doi 1969, 1972) that is the largest work on *Hypocrea* ever published. In it, the anamorphs of many species of *Hypocrea* were described and illustrated. Doi and collaborators described many new species of *Hypocrea* and their anamorphs based on isolates collected in Japan, New Guinea, and South America (Doi 1966, 1968, 1969, 1971, 1972, 1973a, b, 1975, 1976, 1978, Doi & Doi 1980, Doi & Yamatoya 1989).

Doi proposed an infrageneric taxonomy for *Hypocrea* based in large part on anatomy of the stroma, and to a lesser extent on anamorphs and ascospore pigmentation. Based mainly on teleomorph characters, Doi described two subgenera: *H.* subg. *Heterocrea* and *H.* subg. *Hypocrea*. Within *H.* subg. *Hypocrea*, two sections were defined: *H.* sect. *Homalocrea* and *H.* sect. *Hypocrea*. Doi (1972) recognized *H.* subsect. *Creopus* within *H.* sect. *Hypocrea*, in which he included mostly *Hypocrea* species with green ascospores. Within this subsection, he distinguished three "artificial groups" of species, viz. AUREOVIRIDIS, CORNEA and GELATINOSA, based on types of stroma and conidiophore branching. The AUREOVIRIDIS group included species with friable stromata, generally without perithecial protuberances and with trichoderma- or pachybasium-like (Bissett 1991a, b) anamorphs. The CORNEA group was characterized by coriaceous stromata and verticillium-like anamorphs. The GELATINOSA group was characterized by friable stromata, generally with protuberances and gliocladium-like anamorphs. Recent studies based on classical and molecular techniques, have shown that *Hypocrea* species with trichoderma-, pachybasium-, gliocladium-, or verticillium-like anamorphs do not form monophyletic groups (Kindermann *et al.* 1998, Kullnig-Gradinger *et al.* 2002). Considerable evolutionary convergence occurs in the phenotype of the teleomorph, and this phylogenetic study does not support

Doi's subdivisions of *Hypocrea* (Lieckfeldt *et al.* 1998a, Chaverri *et al.* 2000, Kullnig-Gradinger *et al.* 2002).

Most *Hypocrea* species were described in the 19th and early 20th centuries, often based on collections made by travelers, missionaries, pathologists or others in exotic lands and sent to experts such as M.J. Berkeley in England, P. Hennings in Germany, and J.B. Ellis in the United States. As was typical at the time, these newly described species often were published in larger works that included a wide diversity of fungi. None of the descriptions were illuminating, comprising at best a few lines of descriptive text and a note about the substratum. The better descriptions included ascospore measurements, but no effort was made to link collections to anamorphs or to describe stromatal anatomy. The systematic use of teleomorph anatomy by first Dingley and then Doi represented a significant move beyond these brief descriptions. The teleomorph is, after all, the element upon which the system for classifying Ascomycota is based, and it is considered to be the 'name-bearing' part of the holomorph (*i.e.* teleomorph + anamorph) under Article 59 of the International Code of Botanical Nomenclature. However, with increasing efforts to link teleomorphs and anamorphs in the Ascomycota, including *Hypocrea* and *Trichoderma* morphs, and with the introduction of molecular phylogenetics into *Hypocrea* taxonomy, it has become apparent that the *Hypocrea* stroma possesses a highly conserved structure and is of limited use in species-level classification. For example, Samuels *et al.* (1998), in their monograph of the *Hypocrea schweinitzii* (Fr.) Sacc. Complex, showed that teleomorphs of *H. schweinitzii sensu lato* were almost indistinguishable from each other. Differences among species were seen, however, in anamorphs, growth characteristics and DNA sequences. In cases involving closely related species, significant differences are more likely to be visible in the morphology of the *Trichoderma* anamorphs than in the anatomy or morphology of the teleomorph.

In general, *Trichoderma* species have hyaline phialides formed on exposed fertile branches or 'conidiophores', and conidia that are generally smooth, rarely ornamented, typically ellipsoidal to nearly oblong, rarely globose, and mostly green or hyaline, rarely yellow. If chlamydospores are formed, they are typically globose to subglobose and are formed within or at the tips of hyphae. In *T. stromaticum* Samuels *et al.*, chlamydospores are formed that consist of balls of cells; typical chlamydospores are also formed (Samuels *et al.* 2000). *Trichoderma virens* (Miller *et al.*) Arx is characterized in part by the great abundance of chlamydospores that form in pure culture (Chaverri *et al.* 2001a).

Persoon (1794) originally described *Trichoderma* as a Gasteromycete. Only one of the small number of species originally placed in the genus, namely *T. viride*, remains there today. Later, the Tulasne brothers (Tulasne & Tulasne 1860) and Brefeld (1891) proved the link between *T. viride* and *H. rufa*. The Tulasne brothers illustrated *H. rufa* and *T. viride* as one species, following hyphae from the *Hypocrea* stroma to the *Trichoderma* conidiophores. Brefeld isolated single ascospores of *H. rufa* and obtained *T. viride* in culture. Bisby (1939) could not distinguish the anamorph and teleomorph of *H. rufa* from those of *H. gelatinosa*, stating that *H. gelatinosa* "is only a growth form or mature condition of *H. rufa*." Bisby reduced *Trichoderma* to a single species, *T. viride*. *Trichoderma* has been linked only to sexual stages in *Hypocrea,* and to some species of *Podostroma* and *Sarawakus*. The anamorph of the type species of *Podostroma, P. alutaceum* (Pers. : Fr.) Atk., is verticillium-like and not typical of *Trichoderma*, although some unidentified species of the genus have typical *Trichoderma* anamorphs. No anamorph has been linked to the type species of *Sarawakus*, *S. lycogaloides* (Berk. & Broome) Lloyd. Samuels & Rossman (1992) transferred two species from *Hypocrea* to *Sarawakus* because of their formation of eight unicellular ascospores in each ascus. These species have *Trichoderma* anamorphs but, apart from the unicellular ascospores, they are not morphologically consistent with *S. lycogaloides*. With few exceptions, *e.g. H. pallida, H. lutea* (Tode) Petch, and *H. sulphurea* (Schwein.) Sacc., *Hypocrea* species have anamorphs that are morphologically typical of *Trichoderma*.

Rifai (1969) divided *Trichoderma* species into nine "species aggregates," namely, *T. aureoviride* Rifai, *T. hamatum* (Bonord.) Bain., *T. harzianum* Rifai, *T. koningii* Oudem., *T. longibrachiatum* Rifai. *T. piluliferum* Rifai , *T. polysporum* (Link : Fr.) Rifai, *T. pseudokoningii* Rifai, and *T. viride*. Each species aggregate was regarded as possibly comprising more than one morphologically cryptic species. Rifai concluded that anamorph characters alone might not provide a useful taxonomy of *Trichoderma*.

Bissett (1991a) discussed the implications of basing a classification of *Trichoderma* on Rifai's species aggregates. Bissett stated that five of Rifai's aggregates (*i.e. T. harzianum, T. longibrachiatum, T. piluliferum, T. polysporum*, and *T. pseudokoningii*) were apparently narrowly defined species, whereas the remaining aggregates could be interpreted as having relatively large numbers of species. For example, he suggested that more than 20 distinct species could be assigned to each of the morphological species *T. hamatum, T. koningii*, and *T. viride* (Bissett 1984, 1991a, b, c, 1992). Bissett (1984, 1991a, b, c, 1992) recognized five sections: *T.* sect. *Hypocreanum* Bissett, *T.* sect. *Longibrachiatum* Bissett, and *T.* sect. *Pachybasium* (Sacc.) Bissett; *T.* sect. *Saturnisporium* Yoshim. Doi *et al.*, and *T.* sect. *Trichoderma*. The

sections were distinguished by differences in conidiophore branching patterns, phialides, and conidia. *Trichoderma* sect. *Trichoderma* (type species*: T. viride*) is distinguished by producing anamorphs that have narrow and flexuous conidiophores and branches with 2–3 phialides per verticil. The phialides are generally lageniform and are attached to the conidiophore at wide angles, and the conidia are smooth or ornamented. Anamorphs with *T.* sect. *Trichoderma* morphology are often referred in the literature as trichoderma-like. Bissett (1991a) placed *T. koningii* and the *Trichoderma* states corresponding to *Hypocrea rufa/Trichoderma viride*, *H. aureoviridis/T. aureoviride* and *H. atroviridis/T. atroviride* in sect. *Trichoderma.* Several studies have shown that some of the species included by Bissett in *T.* sect. *Pachybasium*, including the neotype of *T. hamatum*, cluster with species in *T.* sect. *Trichoderma* (Kindermann *et al.* 1998, Dodd *et al.* 2000, Lieckfeldt *et al.* 2001, Dodd *et al.* 2002, Kullnig-Gradinger *et al.* 2002). These same studies have also shown that *H. aureoviridis/T. aureoviride* and *H. rufa/T. viride* are not closely related.

Trichoderma sect. *Longibrachiatum* is distinguished by having aggregated conidiophores forming weakly developed pustules. The conidiophores consist of long primary branches and short, unbranched secondary branches. The phialides are solitary, rarely in verticils, and they produce ellipsoidal to oblong, green conidia. One characteristic of the group is the formation of 'intercalary phialides' (*i.e.* short, spur-like phialidic openings that form below the septum delimiting a terminal phialide). These are also found in some other groups but are not nearly as common or as conspicuous as in sect. *Longibrachiatum*. The *H. schweinitzii* Complex, which coincides with species producing anamorphs in *T.* sect. *Longibrachiatum*, was shown to be a monophyletic group based on morphological, cultural and molecular sequence data (Kuhls *et al.* 1997, Samuels *et al.* 1998). Species such as *H. jecorina/T. reesei* and *H. schweinitzii/T. citrinoviride* are examples of taxa within this complex. All known species in the *H. schweinitzii* Complex have colourless ascospores. *Trichoderma* sect. *Saturnisporium* was originally distinguished from other sections by its tuberculate conidia; however, molecular sequence data showed that it was nested within the *H. schweinitzii* Complex (Kuhls *et al.* 1997, Samuels *et al.* 1998).

Trichoderma sect. *Hypocreanum* is characterized by having verticillium- and acremonium-like anamorphs. Bissett (1991a) considered the anamorph of *H. lactea* (Fr. : Fr.) Fr. to be representative of *T.* sect. *Hypocreanum*. Preliminary data show that species closely related to *H. lactea*, such as *H. citrina* (Tode : Fr.) Fr.*, H. pulvinata* Fuckel, and *H. sulphurea* (Schwein.) Sacc., all have anamorphs with *T.* sect. *Hypocreanum* morphology (B.E. Overton, unpubl. data). This type of morphology, however, has been shown to be polyphyletically distributed. *Hypocrea citrina* was studied in detail by Canham (1969). *Hypocrea tawa* Dingley, *T. hamatum, T. pubescens* Bissett, *H. poronioidea* A. Möller, and *H. strictipilosa*/*T. strictipile,* which are not related to *H. lactea,* also have acremonium- or verticillium-like anamorphs and synanamorphs (Samuels & Lodge 1996, Kullnig-Gradinger *et al.* 2002, Chaverri *et al*. 2003a). *Verticillium* Nees *s. str.* is characterized by primary branches rebranching once or a few times and then terminating in verticils of long, subulate phialides. The verticillium-like anamorphs of *Hypocrea/Trichoderma* are distinguished from true *Verticillium* by the presence of a dendritic conidiophore branching pattern, which generally includes secondary branches. *Verticillium luteoalbum* (Link : Fr.) Subram., the type species of the genus, does not belong in the *Hypocreales*, but is rather closely related to *Glomerella* Spauld. & H. Schrenk (Zare *et al.* 2000).

Trichoderma sect. *Pachybasium*, in its morphological sense, so far contains the anamorphs of the majority of the described *Hypocrea/Trichoderma* species. Species in this section often produce compact conidiogenous pustules with branching generally in a pyramidal pattern, with or without fertile or sterile conidiophore elongations, and with phialides typically short and wide, generally produced in crowded clusters of 2–7. Saccardo (1885) erected the genus *Pachybasium* to accommodate *T. hamatum* and *T. hamatum* var. *candidum* Sacc., a species with white conidia (a synonym of *T. hamatum* var. *candidum* = *T. polysporum*). Bissett (1991a) expanded the concept of *Pachybasium* to include species with similar conidiophores and phialides, and elevated the genus *Pachybasium* to a section within *Trichoderma*. Bissett (1991b) also included species consisting of or possessing gliocladium-like anamorphs, *e.g. T. virens, T. flavofuscum* Bissett, *H. gelatinosa*, and *H. lutea* (Tode) Petch, in his concept of *T.* sect. *Pachybasium*. *Trichoderma* sect. *Pachybasium* has since been shown to be polyphyletic (Kindermann *et al.* 1998, Lieckfeldt & Seifert 2000, Kullnig-Gradinger *et al.* 2002). Kindermann *et al.* (1998) divided *T.* sect. *Pachybasium* into two distinct phylogenetic groups termed "A" and "B"; further studies support this division (Kullnig-Gradinger *et al.* 2002, Chaverri *et al.* 2003a). Group A included species, such as *T. atroviride* P. Karsten, *T. hamatum* (the type species of *T.* sect. *Pachybasium*), *T. koningii, T. minutisporum* Bissett, *T. piluliferum* Webster & Rifai in Rifai, *T. polysporum* (Link : Fr.) Rifai, *T. pubescens* Bissett, *T. strigosum* Bissett, and *T. viride*; and group B included *T. crassum* Bissett, *T. fertile* Bissett, *T. flavofuscum, T. harzianum* Rifai, *T. longipile* Bissett, *H.*

semiorbis (Berk.) Berk., *T. spirale* Bissett, *H. strictipilosa/T. strictipile, T. tomentosum* Bissett, and *H. virens/T. virens*. Section *Pachybasium* is to be reduced to a descriptive term, but should not be used as a formal section.

One of the main characteristics of *Trichoderma* sect. *Pachybasium* is that phialides are clustered in heads, often arising from a broad cell. Bissett (1991b) included species in the section that had previously been placed in *Gliocladium* (*e.g. T. virens, T. flavofuscum*) or that, based on classical morphological criteria, could have been included in *Gliocladium* (*e.g.* the anamorph of *H. gelatinosa*). Seifert (1985) and other authors (Rehner & Samuels 1994) found that *Hypocrea* species with gliocladium-like anamorphs are phylogenetically distinct from the type of *Gliocladium*. Closer examination of their morphology shows that all of them can be readily excluded from *Gliocladium*, but not all of them are phylogenetically consistent with *Trichoderma*. DNA sequence analyses indicates that *T. virens* (= *Gliocladium virens*) is a species of *Trichoderma*; it is the anamorph of *H. virens* Chaverri & Samuels (Chaverri *et al.* 2001a) and is closely related to *T. harzianum* (Chaverri *et al.* 2003a). The *Gliocladium viride* Matr. anamorph of *H. lutea* is phylogenetically a member of *Trichoderma* but its gliocladium-like conidiophore is atypical of the genus. The anamorphs of *H. pallida* and related species (Doi & Yamatoya 1989) are virtually indistinguishable from *Gliocladium penicillioides*, the type species of *Gliocladium* and the anamorph of *Sphaerostilbella aureonitens*. Sequence analysis excludes *H. pallida* from *Hypocrea* and places it closer to *Hypomyces* and *Sphaerostilbella* than it is to *H. rufa* (Rehner & Samuels 1994, Põldmaa *et al.* 1999). In addition to *H. virens*/*T. virens*, *H. gelatinosa*, and *H. lutea/G. viride,* a number of unrelated *Hypocrea* species have gliocladium-like anamorphs, including *H. psychrophila* Müller *et al.*, *H. luteovirens* Yoshim. Doi, *H. argillacea* Phill. & Plowr., and *T. crassum* Bissett, among others.

The introduction of DNA sequencing and cladistic analysis of DNA sequences in the early 1990's opened a new era for fungal systematics by providing a new set of independently derived data that could be analyzed in tandem with more classically derived data. The upshot of the molecular revolution has been the ability to understand interrelationships among taxa at all levels. The first DNA-based phylogenetic studies that included genera of the *Hypocreales* analyzed the relationship among different ascomycetes based on ribosomal DNA sequence data (Spatafora & Blackwell 1993, Rehner & Samuels 1994, 1995). As a result of these studies, it was shown that there were four monophyletic clades in the *Hypocreales*: *Bionectria*, *Nectria*, *Claviceps* and *Hypocrea*, now known as the families *Bionectriaceae*, *Nectriaceae*, *Clavicipitaceae* and *Hypocreaceae*, respectively (Rossman *et al.* 1999). Within this group, only the *Hypocrea* and *Bionectria* clades were well supported statistically. The *Hypocrea* clade included *Hypocrea*, *Hypomyces* and *Sphaerostilbella*. As mentioned above, more recent molecular studies have shown that the order *Hypocreales* could be defined to include the clades *Bionectria* (*Bionectriaceae*), *Claviceps* (*Clavicipitaceae*), *Hypocrea* (*Hypocreaceae*), *Nectria* (*Nectriaceae*), and *Niesslia* (*Niessliaceae*).

Certain subgroups within the *Hypocreales* have been studied in detail in recent years. Lieckfeldt *et al.* (1998a) reviewed the progress in molecular systematics of the *Hypocreales*, with emphasis on *Hypocrea* and *Trichoderma.* Lieckfeldt & Seifert (2000) summarized the value of ITS sequences in the taxonomy of the *Hypocreales*. Põldmaa *et al.* (1999) studied phylogenetic relationships in *Hypomyces* and allied genera and Põldmaa (2000) evaluated the generic delimitation of fungicolous *Hypocreaceae*. Phylogenetic relationships of species of *Hypocrea*/*Trichoderma* have also been studied. The first accounts were gene genealogies based on a single gene region, viz. nuclear ribosomal internal transcribed spacers (ITS) DNA. For example, Lieckfeldt *et al.* (1998b) and later Dodd *et al.* (2000) studied the relationship of several species of *Hypocrea/Trichoderma*. Kuhls *et al.* (1997) studied the phylogenetic relationships of species in *T.* sect. *Longibrachiatum*. Samuels *et al.* (1998) monographed the *H. schweinitzii* Complex, which included *T.* sect. *Longibrachiatum*, and also presented a phylogeny. Kindermann *et al.* (1998) analyzed the phylogeny of species in *T.* sect *Pachybasium*. Lieckfeldt *et al.* (1999) used ITS and morphological data to distinguish the new species *T. asperellum* Samuels *et al.* from *T. viride*, and to show the close relationship between these species. Samuels *et al.* (2000) described a new species of *Trichoderma* used in biocontrol, *T. stromaticum*, and presented its phylogenetic relationship to other biocontrol species (*T. harzianum* and *T. virens*). Lieckfeldt *et al.* (2001) studied the phylogenetic position and morphological characterization of *H. aureoviridis*/*T. aureoviride*.

As the development of analytical techniques for phylogenetic sequence analysis progressed, new methods to recognize species based on multiple genes emerged. Lieckfeldt *et al.* (2000) used the endochitinase gene (ECH42) to show phylogenetic relationships, mostly of species in *T.* sect. *Trichoderma*. Samuels *et al.* (2002) used ITS rDNA and translation elongation factor 1-alpha (EF-1α) to study the systematics of two very closely related species, *T. harzianum* and *T. aggressivum* Samuels & W. Gams. Dodd *et al.* (2002) studied the phylogenetic relationship of two morphologically similar species, *H. patella* Cooke & Peck and *H. neorufa* Pat., using ITS

rDNA and EF-1α. Kullnig-Gradinger *et al.* (2002) presented a multigene phylogeny of many species of *Hypocrea/Trichoderma* using 28S and ITS rDNA, small subunit (SSU) mitochondrial (mt) ribosomal DNA (rDNA), EF-1α, and ECH42.

Analyses of phenotype including anamorphs, teleomorphs, and growth rates, in combination with molecular sequence data, has contributed to our understanding of the evolution of *Hypocrea/Trichoderma* and has helped to show link between particular anamorphs and teleomorphs. Samuels and collaborators included multiple morphological characters, growth data and DNA analyses in their monograph of the *H. schweinitzii* Complex (Samuels *et al.* 1998). Similar combination studies were performed in the study of the "green mold" of commercial mushrooms, *T. aggressivum*, and its relationship to *T. harzianum* (Samuels *et al.* 2002); in distinguishing morphologically similar species (Lieckfeldt *et al.* 1999, Samuels *et al.* 1999, Dodd *et al.* 2002); and in showing the relationships of species in *T.* sect. *Pachybasium* with conidiophore elongations (Chaverri *et al.* 2003a). These studies have added more than 20 newly described species of *Hypocrea* and *Trichoderma*.

In addition to showing phylogenetic relationships among species, studies in which phenotype and DNA analyses were combined have tended to confirm the monophyly of species distinguished by Bissett (1984, 1991a, b, c, 1992) on morphological grounds. In some instances, however, even those species have been shown to comprise more than one species. Lieckfeldt *et al.* (1999) and Samuels *et al.* (1999) demonstrated that the type species of *Trichoderma*, *T. viride,* comprised at least two species, one of which was described as *T. asperellum*. *Trichoderma viride* is mainly found in the northern hemisphere, whereas *T. asperellum* is ubiquitous in both mesic and xeric soil habitats in regions as diverse as Russia, Central Africa and Saudi Arabia. The residual *T.viride* is still paraphyletic. The current morphological species concept of *T. koningii* is also polyphyletic, comprising at least four phylogenetic species (G. J. Samuels, unpubl. data). Both *T. viride* and *T. koningii* may be conceived of as species complexes, as discussed below. On the other hand, the cosmopolitan species *H. atroviridis/T. atroviride* is monophyletic (Dodd *et al.* 2003).

Surprisingly, *T. harzianum*, a ubiquitous species that has been thought to be paraphyletic (*e.g.* Grondona *et al.* 1997) because of its seemingly great phenotypic and genotypic variability, has been shown to be a monophyletic 'species complex,' that is, a species that comprises multiple phenotypically indistinguishable evolutionary lines. High infraspecific variation has been found in *H. lixii*/*T. harzianum* in studies using multiple morphological, physiological and molecular characters (Grondona *et al.* 1997, Hermosa *et al.* 2000, Lee & Hseu 2002, Samuels *et al.* 2002, Chaverri *et al.* 2003b). Multigene phylogenetic analyses have revealed that species complexes in *Trichoderma* are common. Besides *H. lixii/T. harzianum,* the aforementioned *H. rufa/T. viride* and *H. koningii*/*T. koningii* groups may also be thought of as representing species complexes. Samuels *et al.* are in the process of revealing multiple species within the *H. rufa/T. viride* and *H. koningii*/*T. koningii* complexes (G. J. Samuels unpubl.). In addition, a degree of morphological variation has been encountered in specimens and isolates of *Hypocrea* cf. *chlorospora* B. & M.A. Curtis. In the present study, the close phylogenetic and morphological relationships of these specimens and isolates are examined to determine if they constitute a species complex.

In order to develop a natural taxonomic system for ascomycetous fungi that reflects the full biology (*e.g.* the life cycles) of these organisms, the delineation of anamorph-teleomorph relationships is a major goal of fungal systematics. The combination of phenotypic and DNA sequence data has also been proven useful for making such connections in *Hypocrea/Trichoderma*. The first anamorph-teleomorph connection made using DNA analyses was made by Kuhls *et al.* (1996), who showed that the asexual industrial fungus *Trichoderma reesei* E.G. Simmons was the anamorph of *Hypocrea jecorina* Berk. & Broome. Samuels *et al.* (1998), in their monograph of the *H. schweinitzii* Complex, showed the connection of *H. pseudokoningii* and *T. pseudokoningii, H. schweinitzii* and *T. citrinoviride*, and *H. orientale* and *T.* cf. *longibrachiatum*. Additional links of *Hypocrea* species to named *Trichoderma* species are presented in Table 2. In the present paper the previously unknown teleomorph of *T. crassum* is described. In addition, anamorphs of species of *Hypocrea* with green ascospores are named and described.

The objectives of the present study were: 1) to describe or re-describe species of *Hypocrea* with green ascospores; 2) to determine if species of *Hypocrea* with green ascospores form a monophyletic group; 3) to determine and describe monophyletic groups that exclusively contain species with green ascospores; and 4) to describe *Hypocrea* species complexes that include at least some species with green ascospores (*e.g. H.* cf. *chlorospora* and *H. lixii/T. harzianum* complex). To accomplish these objectives, multiple phenotypic characters were evaluated, and phylogenetic analyes were performed based on sequences of two genes, the RNA polymerase II subunit (RPB2) and EF-1α. Descriptions and illustrations of 40 species of *Hypocrea* and their known *Trichoderma* anamorphs are presented as well as keys to these species.

Table 2. Anamorph-teleomorph connections to named species of *Hypocrea/Trichoderma.**

Teleomorph	Anamorph	Source
H. atroviridis	*T. atroviride*	Dodd *et al.* 2003
H. aureoviridis	*T. aureoviride*	Lieckfeldt *et al.* 2001
H. jecorina	*T. reesei*	Kuhls *et al.* 1996
H. koningii	*T. koningii*	Lieckfeldt *et al.* 1998b
H. lixii	*T. harzianum*	Chaverri & Samuels 2002; Chaverri *et al.* 2003b
H. lutea	*Gliocladium viride*	Domsch *et al.* 1980
H. minutispora	*T. minutisporum*	Lu *et al.* 2003
H. pachybasioides	*T. polysporum*	Doi 1972; Bissett 1991b; Lu *et al.* 2003
H. pilulifera	*T. piluliferum*	Rifai 1969
H. pseudokoningii	*T. pseudokoningii*	Samuels *et al.* 1998
H. rufa	*T. viride*	Tulasne & Tulasne 1860
H. schweinitzii	*T. citrinoviride*	Samuels *et al.* 1998
H. strictipilosa	*T. strictipile*	Chaverri *et al.* 2003a
H. virens	*T. virens*	Chaverri *et al.* 2001a

* Species newly described in this study are not included in the table.

Ecology and economic importance of *Hypocrea/Trichoderma*

Species of *Hypocrea/Trichoderma* typically grow on decaying woody substrata but also on other fungi, including ascomycetes and basidiomycetes, and rarely on herbaceous tissues. *Trichoderma* is universally found as a soil fungus.

There is usually no apparent host specialization (Rossman *et al.* 1999). There are, however, some exceptions to this trend. *Hypocrea pulvinata* Fuckel occurs only on aphyllophoraceous basidiomycetes, *e.g. Tyromyces*, *Fomitopsis,* and *Piptoporus*, while *H. latizonata* Peck is restricted to *Cyathus*, *H. avellanea* Carey & Rogerson to *Gymnopus subnudus*, *H. pallida* to members of the *Aphyllophorales sensu lato* and *H. spinulosa* Fuckel to grasses growing in alpine and boreal regions. *Hypocrea psychrophila* has only been found growing on *Rhododendron ferrugineum* and *Vaccinium myrtillus* in the Swiss Alps (Müller *et al.* 1972). *Trichoderma stromaticum* Samuels *et al.* and its *Hypocrea* teleomorph are only known from species of cocoa (genus *Theobroma*, family *Sterculiaceae)* and are often associated with tissue infected with the basidiomycetous pathogen *Crinipellis perniciosa*. *Trichoderma aggressivum* Samuels *et al.* has so far been found only on *Agaricus bisporus* in commercial mushroom production. Because *Hypocrea* stromata are most often found on decaying wood that is inhabited by other fungi, it is not possible to tell whether the *Hypocrea* is obtaining its nutrients at the expense of another fungus or the decaying wood or both.

Some species show biogeographical specialization. *Hypocrea jecorina/Trichoderma reesei* is known only from the equatorial zone, while *H. semiorbis* (Berk.) Berk. is known only from New Zealand and Australia and *H. aureoviridis/T. aureoviride, H. pilulifera/T. piluliferum, H. placentula* W.B. Grove are found only in northern Europe and the United Kingdom. *Trichoderma stromaticum* naturally occurs only in the Amazon basin (Samuels *et al.* 2000) and *H. nigrovirens* Chaverri, Samuels & E.L. Stewart has been found only in Costa Rica (Chaverri *et al.* 2001b). *Hypocrea minutispora/T. minutisporum* is known only from north temperate collections and *H. pachybasioides/T. polysporum* is cosmopolitan at north and south temperate latitudes but has never been found in tropical regions ("tropical" defined as occurring between the Tropics of Cancer and Capricorn) (Lu *et al.* 2003).

Hypocrea/*Trichoderma* species are economically important because of the effect that some of them have on disease-causing fungi. This antifungal activity has been extensively documented and suggestions have been made that the biocontrol effects exerted by these fungi may be based on one or more of three possible factors, namely antibiosis (Howell & Stipanovic 1983, 1991, Lumsden *et al.* 1992, Ghisalberti & Rowland 1993, Sawa *et al.* 1994, Elad 2000a, Kexiang *et al*. 2002), mycoparasitism (Elad 2000a, Mishra *et al.* 2000, Viterbo *et al.* 2002), and nutrient competition (Simon & Sivasithamparam 1989). The volume edited by Harman & Kubicek (1998) contains several papers that include comprehensive information about *Hypocrea/Trichoderma* enzymes, biological control and commercial applications. Green-ascospore-producing species of *Hypocrea/Trichoderma* that have known antifungal activities are *H. virens/T. virens* and *H. lixii/T. harzianum*. In addition, *T. harzianum* and *T. virens* are the active ingredients in the commercial preparations, TRICHODEX™ and SoilGard™, respectively, used in biological control of various fungal diseases (Ricard 1981, Harman 1990, Lumsden *et al.* 1993, Harman & Lo 1996).

Trichoderma harzianum has been found to be effective against many phytopathogens, and is considered a generalist biocontrol agent. For example, it is effective against *Rhizoctonia* damping-off in radish, corn, soybean, cucumber, and sugar beet (Lewis & Papavizas 1980, Kommedahl *et al.* 1981, Lifshitz *et al.* 1985, Dutta & Das 1999, Mishra *et al.* 2000), *Sclerotium rolfsii* plant wilt (El-Katatny *et al.* 2000, Mishra *et al.* 2000), grey-mold diseases caused by *Botrytis cinerea* (Harman *et al.* 1995, Elad & Kapat 1999, Elad 2000b, Kovach *et al.* 2000, Hjeljord *et al.* 2001), take-all disease in wheat (Ghisalberti & Sivasithamparam 1991), *Botryosphaeria berengeriana* f. sp. *piricola* apple ring-rot (Kexiang *et al.* 2002), *Armillaria* spp. root-rot (Otieno *et al.* 2003a, b), *Venturia inaequalis* apple scab (Bolar *et al.* 2000, Otieno

et al. 2003a, b), *Fusarium udum* wilt of pigeon pea (Prasad *et al.* 2002), and cacao witches' broom caused by *Crinipellis perniciosa* (de Azevedo *et al.* 2000, De Marco *et al.* 2000). *Trichoderma harzianum* has also been useful in the control of root diseases caused by nematodes, *e.g.* strawberry black root-rot caused by *Pratylenchus penetrans* and *Rhizoctonia fragariae* (LaMondia & Cowles 2002) and root knot caused by *Meloidogyne javanica* (Sharon *et al.* 2001). It has also been found to enhance plant growth and disease resistance upon plants (Inbar *et al.* 1994, Gromovich *et al.* 1998, Yedidia *et al.* 2001).

Trichoderma virens has also been an effective biocontrol agent against many plant pathogens. For example, it is useful against *Phytophthora erythroseptica* pink rot of potato and root and stem rot of tomato (Etebarian *et al.* 2000), damping-off diseases of various types of seedlings (Roberts & Lumsden 1990, Howell & Stipanovic 1991, Lumsden *et al.* 1996, Harris & Lumsden 1997), black-pod of cacao caused by *Phytophthora palmivora* (Krauss & Soberanis 2001, 2002), gladiolus corm rot and wilt caused by *Fusarium oxysporum* f. sp. *gladioli* (Mishra *et al.* 2000), *Sclerotinia sclerotiorum* infection of beans (Huang *et al.* 2000), and the root-knot nematode *Meloidogyne incognita* on bell pepper (Meyer *et al.* 2001). *Trichoderma virens* was reported as having growth-promoting activities when it was cultivated with rice seedlings (Mishra *et al.* 2000). The mycotoxins gliotoxin, viridin, and gliovirin have been linked to the antifungal capabilities of *T. virens* (Weindling & Emerson 1936, Weindling 1937, 1941, Howell & Stipanovic 1983, DiPietro *et al.* 1993). *Trichoderma flavofuscum*, which we consider a synonym of *T. virens*, also produces these mycotoxins (Avent *et al.* 1993). Gliotoxin is also formed by *Aspergillus fumigatus* Fresen. (Nieminen *et al.* 2002, Wenehed *et al.* 2003). Peptaibol metabolites produced by some *Trichoderma* species have also been linked to antifungal action (Schirmbock *et al.* 1994, Oh *et al.* 2002). Other secondary metabolites, including mycotoxins, produced by *Hypocrea*/*Trichoderma* were listed by Sivasithamparam & Ghisalberti (1998).

In addition to the use of *Hypocrea/Trichoderma* species in the biological control of plant pathogenic fungi, many species have the ability to break down cellulosic materials through the production of cellulases. This ability has led to the commercial exploitation of some *Hypocrea/Trichoderma* species in production of enzymes used in manufacture of clothes-washing detergent, animal feed and fuel (Reese & Mandels 1989, Kubicek *et al.* 1990, Nsereko *et al.* 2002). One unusual application of an unidentified species of *Trichoderma* is in the bioconversion or biodegradation of domestic wastewater sludge into compost (Molla *et al.* 2002). Properties of other species that might be useful in bioremediation have also been investigated. Several studies have shown the potential of *T. harzianum* to bioremediate soils contaminated with pesticides such as pentachlorophenol and endosulfan (Katayama & Matsumura 1993, Rigot & Matsumura 2002).

With regard to biosafety concerns, *Hypocrea*/*Trichoderma* species are not known to cause disease in healthy humans; however, there are numerous reports documenting pathogenicity in humans with immunocompromising conditions (Loeppke *et al.* 1983, Jacobs *et al.* 1992, Kuhls *et al.* 1999). One case has been reported in which *T. harzianum* caused a fatal disseminated infection in a renal transplant patient (Guarro *et al.* 1999).

MATERIALS AND METHODS

Isolates and herbarium specimens

The isolates and specimens investigated are listed with the description of each species. Isolates used for DNA analyses are listed in Table 3 with their corresponding GenBank accession numbers. The majority of isolates were obtained from fresh collections of *Hypocrea*, but others were obtained from Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS) and from Agriculture and Agri-Food Canada, Eastern Cereals and Oilseeds Research Centre, Ottawa, Canada (DAOM). Representative isolates have been deposited in American Type Culture Collection, Manassas, VA, USA (ATCC) as well as CBS and DAOM. Single-ascospore isolations from fresh collections of *Hypocrea* were made on cornmeal dextrose medium (CMD), consisting of Difco cornmeal agar (Difco Laboratories, Detroit, MI, USA) + 2% dextrose + 1% antibiotic solution (0.2% Sigma [Sigma-Aldrich Corp., St. Louis, MO, USA] Streptomycin Sulfate + 0.2% Sigma Neomycin Sulfate + distilled water). A micromanipulator was used to obtain single propagules for inoculation. The cultures obtained are maintained at the U.S. National Fungus Collection (BPI) on Difco cornmeal agar (CMA) slants at 8 ºC and in liquid nitrogen in cryovials with 10% glycerol.

The majority of herbarium specimens examined are kept in BPI, but others, such as type specimens, were obtained from William and Lynda Steere Herbarium (NY), the Herbarium of the Royal Botanic Gardens, Kew (K), the New Zealand Fungal Herbarium (PDD), the Farlow Reference Library and Herbarium of Cryptogamic Botany (FH), the Museum of Evolution, Botany Section, Uppsala University (UPS), the University of Sheffield Botany Department Herbarium (SHD), the Herbarium of Mycology and Plant Pathology Department, Agharkar Research Institute (AMH), the Department of Botany Herbar-

ium of INBio, the National Biodiversity Institute of Costa Rica (INB), the Herbarium Conservatoire & Jardin botaniques de la Ville de Genève (G), and others. The frequently cited collectors G.J. Samuels and C.T. Rogerson are abbreviated as G.J.S. and C.T.R.

Table 3. List of isolates studied, geographic origin and Genbank accession numbers.

			GenBank numbers	
Species	**Isolate**	**Geographic origin**	**EF-1α**	**RPB2**
Hypocrea aureoviridis	CBS 245.63*	U.K.	AF534575	AF545509
Hypocrea aureoviridis	G.J.S. 98-23 (= IMI 355906)	U.K.	AY391961	AY391898
Hypocrea avellanea	C.T.R. 77-155	Pennsylvania, U.S.A.	AY225857	AF545562
Hypocrea candida	P.C. 59* (= CBS 114249)	Costa Rica	AY391962	AY391899
Hypocrea catoptron	G.J.S. 02-76 (= CBS 114232)	Sri Lanka	AY391963	AY391900
Hypocrea ceracea	G.J.S. 88-28	New York, U.S.A.	AY391964	AY391901
Hypocrea ceracea	G.J.S. 89-136	North Carolina, U.S.A.	AY391965	AY391902
Hypocrea ceracea	G.J.S. 95-159* (= CBS 114245)	New York, U.S.A	AF534603	AF545508
Hypocrea ceramica	G.J.S. 88-70* (= CBS 114576)	North Carolina, U.S.A.	AF534593	AF545510
Hypocrea chlorospora	G.J.S. 88-33* (= CBS 114231)	Connecticut, U.S.A.	AY391966	AY391903
Hypocrea chlorospora	G.J.S. 91-150	Maryland, U.S.A.	AY391967	AY391904
Hypocrea chlorospora	G.J.S. 95-88	Puerto Rico		AY391905
Hypocrea chlorospora	G.J.S. 98-1	Costa Rica	AY391968	AY391906
Hypocrea chlorospora	P.C. 4	Pennsylvania, U.S.A.	AF534578	AF545515
Hypocrea chromosperma	G.J.S. 90-58	New York, U.S.A.	AY391969	AY391908
Hypocrea chromosperma	G.J.S. 90-59	New York, U.S.A.	AY391970	AY391909
Hypocrea chromosperma	G.J.S. 91-128	Maryland, U.S.A.	AY391971	AY391910
Hypocrea chromosperma	G.J.S. 92-88	Maryland, U.S.A.	AY391972	AY391911
Hypocrea chromosperma	G.J.S. 94-67	Maryland, U.S.A.	AY391973	AY391912
Hypocrea chromosperma	G.J.S. 94-68* (= CBS 114577)	Maryland, U.S.A.	AY391974	AY391913
Hypocrea chromosperma	G.J.S. 95-196	Indiana, U.S.A.	AY391975	AY391914
Hypocrea chromosperma	G.J.S. 98-73	New Jersey, U.S.A.	AY391976	AY391915
Hypocrea cinnamomea	G.J.S. 96-128	Taiwan	AY391977	AY391916
Hypocrea cinnamomea	G.J.S. 96-165	Taiwan		AY391917
Hypocrea cinnamomea	G.J.S. 97-230* (= CBS 114235)	Missouri, U.S.A.		AY391918
Hypocrea cinnamomea	G.J.S. 97-233	Missouri, U.S.A.	AY391978	AY391919
Hypocrea cinnamomea	G.J.S. 97-237	Missouri, U.S.A.	AY391979	AY391920
Hypocrea citrina	CBS 894.85	Belgium	AY225856	AF545561
Hypocrea costaricensis	P.C. 21	Costa Rica	AY391980	AY391921
Hypocrea crassa	G.J.S. 01-227* (= CBS 114230)	Thailand		AY481587
Hypocrea cremea	G.J.S. 02-52	New Zealand	AY391981	AY391922
Hypocrea cremea	G.J.S. 91-125* (= CBS 111146)	New York, U.S.A.	AF534598	AF545511
Hypocrea cuneispora	G.J.S. 91-93* (= CBS 111148)	Virginia, U.S.A.	AF534600	AF545512
Hypocrea estonica	G.J.S. 96-129* (= CBS 111147)	Estonia	AF534604	AF545514
Hypocrea gelatinosa	G.J.S. 88-17	France	AF534579	AF545516
Hypocrea gelatinosa	G.J.S. 93-10	France	AY391982	AY391923
Hypocrea gelatinosa	G.J.S. 98-184* (= CBS 114246)	Austria	AY391983	AY391924
Hypocrea lixii	G.J.S. 90-22	Wisconsin, U.S.A.	AY391984	AY391925
Hypocrea lutea	G.J.S. 89-129	New York, U.S.A.	AF534581	AF545517
Hypocrea megalocitrina	B.E.O. 00-09	North Carolina, U.S.A.	AY225855	AF545563
Hypocrea melanomagna	G.J.S. 99-153* (= CBS 114236)	Australia	AY391985	AY391926
Hypocrea minutispora	CBS 901.72		AY392009	AY481588
Hypocrea nigrovirens	G.J.S. 99-64* (= CBS 114330)	Costa Rica	AF534582	AF545518
Hypocrea pachybasioides	G.J.S. 90-4	Mississippi, U.S.A.	AY392010	AY481589

Hypocrea pezizoides	G.J.S. 01-231	Thailand	AY225859	AF545564
Hypocrea phyllostachydis	G.J.S. 92-123* (= CBS 114071)	France	AF534576	AF545513
Hypocrea phyllostachydis	G.J.S. 92-81	France	AY391986	AY391927
Hypocrea pilulifera	CBS 814.68*	U.K.	AF534583	AF545519
Hypocrea psychrophila	Hy 8	Switzerland	AF534584	AF545520
Hypocrea pulvinata	G.J.S. 98-104	Germany	AY225861	AF545559
Hypocrea rufa	G.J.S. 89-127	North Carolina, U.S.A.	AF534585	AF545521
Hypocrea semiorbis	DAOM 167636	New Zealand	AF545568	AF545522
Hypocrea sinuosa	G.J.S. 90-117	North Carolina, U.S.A.	AY391987	AY391928
Hypocrea sinuosa	G.J.S. 90-131			AY391929
Hypocrea sinuosa	G.J.S. 90-38	Guyana	AY391988	AY391930
Hypocrea sinuosa	G.J.S. 90-41	Connecticut, U.S.A.	AY391989	AY391931
Hypocrea sinuosa	G.J.S. 90-88	North Carolina, U.S.A.	AY391990	AY391932
Hypocrea sinuosa	G.J.S. 91-72	Virginia, U.S.A.		AY391933
Hypocrea sinuosa	G.J.S. 92-79	New York, U.S.A.	AY391991	AY391934
Hypocrea sinuosa	G.J.S. 95-147	New York, U.S.A.	AY391992	AY391935
Hypocrea sinuosa	G.J.S. 95-203	Puerto Rico	AY391993	AY391936
Hypocrea sinuosa	G.J.S. 97-205	France	AY391994	AY391937
Hypocrea sinuosa	G.J.S. 95-206	Puerto Rico		AY391938
Hypocrea sinuosa	G.J.S. 95-209	Puerto Rico		AY391939
Hypocrea sinuosa	G.J.S. 97-221	U.S.A.	AY391995	AY391940
Hypocrea sinuosa	G.J.S. 98-163	France	AY391996	AY391941
Hypocrea sinuosa	P.C. 8* (= CBS 114247)	New York, U.S.A.	AY391997	AY391942
Hypocrea sinuosa	P.C. 9	New York, U.S.A.	AY391998	AY391943
Hypocrea sp. (= *Podostroma*)	G.J.S. 95-28	Puerto Rico	AY392008	AY391960
Hypocrea spinulosa	G.J.S. 99-25	Unknown		AY391944
Hypocrea straminea	G.J.S. 02-84* (= CBS 114248)	Sri Lanka	AY391999	AY391945
Hypocrea strictipilosa	C.T.R. 77-149	New York, U.S.A.	AF534589	AF545523
Hypocrea strictipilosa	C.T.R. 78-201	Denmark	AF534590	AF545524
Hypocrea strictipilosa	G.J.S. 00-170	Russia	AF534591	AF545525
Hypocrea strictipilosa	G.J.S. 00-171	Russia	AF534592	AF545526
Hypocrea strictipilosa	G.J.S. 89-114	Maryland, U.S.A.	AF534595	AF545527
Hypocrea strictipilosa	G.J.S. 89-115	Maryland, U.S.A.	AF534596	AF545528
Hypocrea strictipilosa	G.J.S. 90-64	New York, U.S.A.	AF534597	AF545529
Hypocrea strictipilosa	G.J.S. 91-126	New York, U.S.A.	AF534599	AF545530
Hypocrea strictipilosa	G.J.S. 93-33	France		AY391946
Hypocrea strictipilosa	G.J.S. 93-57	Maryland, U.S.A.	AY392000	AY391947
Hypocrea strictipilosa	G.J.S. 94-114	Estonia		AY391948
Hypocrea strictipilosa	G.J.S. 94-97	France	AF534601	AF545531
Hypocrea strictipilosa	G.J.S. 95-163	Austria	AY392001	AY391949
Hypocrea strictipilosa	G.J.S. 96-130	Estonia	AF534605	AF545532
Hypocrea strictipilosa	G.J.S. 96-162	Indiana, U.S.A.		AY391950
Hypocrea strictipilosa	G.J.S. 96-189	Indiana, U.S.A.	AF534606	AF545533
Hypocrea strictipilosa	G.J.S. 96-190	Indiana, U.S.A.	AF534607	AF545534
Hypocrea strictipilosa	G.J.S. 97-196	Japan		AY391951
Hypocrea strictipilosa	G.J.S. 97-236	Missouri, U.S.A.		AY391952
Hypocrea strictipilosa	G.J.S. 98-110	Germany	AF534608	AF545535
Hypocrea strictipilosa	G.J.S. 98-113	Germany	AF534609	AF545536
Hypocrea strictipilosa	G.J.S. 98-117	Germany	AF534610	AF545537
Hypocrea strictipilosa	G.J.S. 98-91	Pennsylvania, U.S.A.	AF534612	AF545538

Hypocrea sulawesensis	G.J.S. 85-228*	Indonesia	AY392002	AY391954
Hypocrea sulphurea	G.J.S. 95-190	Indiana, U.S.A.	AY225858	AF545560
Hypocrea surrotunda	G.J.S. 88-73* (= CBS 111145)	Connecticut, U.S.A.	AF534594	AF545540
Hypocrea tawa	G.J.S. 02-79	Sri Lanka	AY392003	AY391955
Hypocrea tawa	G.J.S. 97-174* (= CBS 114233)	Thailand	AY392004	AY391956
Hypocrea thailandica	G.J.S. 97-61* (= CBS 114234)	Thailand	AY392005	AY391957
Hypocrea thelephoricola	G.J.S. 95-135* (= CBS 114237)	Maryland, U.S.A.	AY392006	AY391958
Hypocrea virescentiflava	P.C. 278	Costa Rica	AY392007	AY391959
Hypomyces stephanomatis	G.J.S. 88-50	North Carolina, U.S.A.	AF534632	AF545566
Nectria cinnabarina	G.J.S. 91-111	Virginia, U.S.A.	AF534633	AF545567
Trichoderma aggressivum	CBS 100525	United Kingdom	AF534614	AF545541
Trichoderma citrinoviride	G.J.S. 01-364	New York, U.S.A.	AY225860	AF545565
Trichoderma crassum	DAOM 164916* (= CBS 336.93)	Quebec, Canada	AF534615	AF545542
Trichoderma crassum	G.J.S. 95-157	New York, U.S.A.	AF534602	AF545543
Trichoderma fasciculatum	DAOM 167646* (= CBS 118.72)	The Netherlands	AF534616	AF545544
Trichoderma fertile	DAOM 167070	Canada	AF534617	AF545545
Trichoderma fertile	DAOM 167161* (= CBS 339.93)	Canada	AF534618	AF545546
Trichoderma flavofuscum	DAOM 167652* (= CBS 248.59)	Georgia, U.S.A.	AF534619	AF545547
Trichoderma hamatum	DAOM 167057* (= CBS 102160)	Canada	AF534620	AF545548
Trichoderma harzianum	CBS 226.95*	England	AF534621	AF545549
Trichoderma longipile	DAOM 177227* (= CBS 340.93)	Canada	AF534622	AF545550
Trichoderma oblongisporum	DAOM 167085 (= CBS 344.98)	Canada	AF534623	AF545551
Trichoderma pubescens	DAOM 166162* (= CBS 345.93)	North Carolina, U.S.A.	AF534624	AF545552
Trichoderma spirale	DAOM 183974* (= CBS 346.93)	Thailand	AF534626	AF545553
Trichoderma strictipile	DAOM 167072	Canada	AF534627	AF545554
Trichoderma strictipile	DAOM 172827* (= CBS 347.93)	Quebec, Canada	AF534628	AF545555
Trichoderma strigosum	DAOM 166121* (= CBS 348.93)	North Carolina, U.S.A.	AF534629	AF545556
Trichoderma stromaticum	P.C. 209	Brazil	AF534613	AF545539
Trichoderma tomentosum	DAOM 178713A* (= CBS 349.93)	Ontario, Canada	AF534630	AF545557
Trichoderma virens	Gli 39* (= CBS 249.59)	U.S.A.	AF534631	AF545558

* Ex-type culture.

Morphological characterization

Morphological observations of the anamorph were based on cultures grown on CMD in vented plastic Petri plates 9 cm in diam in an incubator at 20 °C, with 12 h fluorescent light and 12 h darkness. Observations were made and digital images taken within approximately one week or when the first green conidia were formed. The following standard characters were noted and measured: type of anamorph (pachybasium-, verticillium-, gliocladium-, or trichoderma-like; see Fig. 1); presence or absence of synanamorphs; width of phialide base, phialide width at the widest point, phialide length and length/width ratio (L/W); width of the cell from which phialides arise (= metula, subtending hypha); conidial colour, length, width, L/W, and ornamentation; presence or absence of chlamydospores, and chlamydospore width. Measurements of continuous characters such as length were made using the beta 4.0.2 version of Scion Image software (Scion Corporation, Frederick, MD, USA). Colony appearance was described from CMD at 20 °C and potato-dextrose-agar (PDA, Difco) at 25 °C, and included observations on the formation, distribution and shape of tufts or pustules. The presence of chlamydospores was recorded by examining the reverse of a colony grown on CMD for *ca*. one week at 20 °C under 12 h darkness and 12 h cool white fluorescent light with 40 × objective of a compound microscope. Colour terminology is from Kornerup & Wanscher (1978).

Herbarium specimens of *Hypocrea* were rehydrated briefly in 3% KOH. Rehydrated stromata were

supported by Tissue-Tek O.C.T. Compound 4583 (Miles Inc., Elkhart, IN, U.S.A.) and sectioned at a thickness of *ca*. 15 µm with a freezing microtome.

The following teleomorph characters were evaluated: diameter, height, colour, shape and surface texture of the stroma; perithecial shape, length and width; reaction to 3% KOH, colour and width of the perithecial wall; ostiolar canal length; colour and 3% KOH reaction of the outer region of the stroma; shape, length and wall thickness of cells of the outer, middle (situated immediately below the outer region) and inner region (situated below perithecia) of the stroma; ascus length and width; distal and proximal part-ascospore length and width.

Confidence intervals ($\alpha = 0.05$), minimum and maximum values for the anamorph and teleomorph morphological characters measured were calculated using Systat 8.0 (SPSS, Inc., Chicago, IL, U.S.A.).

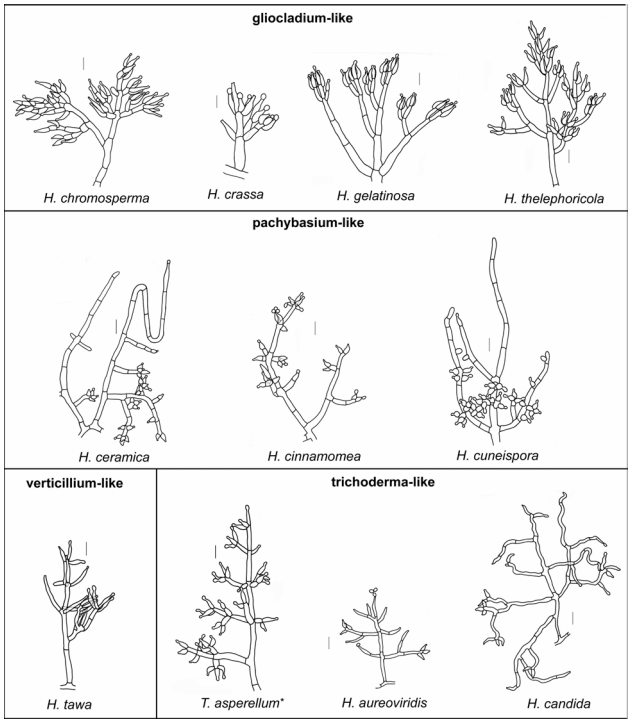

Fig. 1. Selected examples of conidiophore types of the *Trichoderma* anamorphs of *Hypocrea* species with green ascospores. **Trichoderma asperellum*, which belongs in *T.* sect. *Trichoderma s. str.,* does not have a known teleomorph. All known teleomorphs in *T.* sect. *Trichoderma s. str.* have teleomorphs with hyaline ascospores.

Growth and colony characterization

Growth rate and optimum temperature for growth were determined following the protocol of Samuels *et al.* (2002) on PDA and "Synthetischer nährstoffarmer" agar (synthetic nutrient-poor agar, SNA, Nirenberg 1976). Each 9-cm Petri plate contained 20 mL of freshly made media. Five-mm plugs taken from the edge of actively growing colonies were placed 1 cm from the edge of the plate. Colony radius was measured at 24, 48, and 72 h at 15, 20, 25, 30, and 35 °C. Each growth-rate experiment was repeated three times and the results were averaged for each isolate. The time of first appearance of green conidia, the presence of diffusing pigment in the agar, and the colony appearance and odour were also noted.

DNA extraction, polymerase chain reaction (PCR) and sequencing

To obtain fresh mycelia for DNA extraction, the isolates were grown in Difco potato dextrose broth in a 5 cm-diam Petri plate for 3–5 d. The mycelial mat was dried using clean, absorbent paper towels. The entire dried mycelial mat was then placed in a 1.5-mL Eppendorf tube for immediate DNA extraction. Extraction of genomic DNA was done using the Puregene™ Genomic DNA Isolation Kit (Gentra Systems, Minneapolis, MN, U.S.A.). The gene regions studied were RPB2 and EF-1α. Primers were fRPB2-5F (5'-GA(T/C)GA(T/C)(A/C)G(A/T)GATCA(T/C)T T (T/C)GG-3'), and fRPB2-7cR (5'-CCCAT (A/G)GCTTG(T/C)TT(A/G)CCCAT-3') (Liu *et al.* 1999) for the former region and EF1-983F (5'-GC(C/T)CC(C/T)GG(A/C/T)CA(C/T)GGTGA(C/T)T T(C/T)AT-3') (Carbone & Kohn 1999) and EF1-2218R(5'-ATAC(A/G)TG (A/G)GC(A/G)AC (A/G) GT(C/T)TG-3') (S. A. Rehner, pers. comm.) for the latter. PCR reactions were set-up using the following ingredients for each 50 µL reaction: 5 µL of Perkin-Elmer 10 × Buffer with $MgCl_2$ (Applied Biosystems, Branchburg, NJ, U.S.A.), 10 µL of 1 mM dNTPs, 2.5 µL of 10 µM forward primer, 2.5 µL of 10 µM reverse primer, 1 µL of Sigma dimethyl sulfoxide (DMSO), 0.5 µL of Perkin-Elmer AmpliTaq Gold™ Taq Polymerase, a maximum of 25 ng/µL of genomic DNA, and double-distilled water to complete a total of 50 µL per reaction. The PCR reactions were placed in a Bio-Rad iCycler thermocycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.) under the following temperature-cycling parameters: Step 1) 10 min at 95 ºC; step 2) 40 cycles of 30 sec at 94 ºC, followed by 30 sec at 50 ºC for RPB2 or 55 ºC for EF-1α, followed by 1 min at 72 ºC; and step 3) 10 min at 72 ºC. The resulting products were purified with the QIAquick™ PCR Purification Kit (Qiagen, Inc., Valencia, CA, U.S.A.) and QIAquick™ Gel Extraction Kit, when more than one band was amplified. Sequencing was performed at the DNA Sequencing Facility (Center for Agricultural Biotechnology, University of Maryland, College Park, MD, U.S.A.) using Perkin-Elmer Big Dye terminators with dITP (Applied Biosystems) and an Applied Biosystems DNA sequencer model 3100. Sequences were edited and assembled using Sequencher 4.1 (Gene Codes, Madison, WI, U.S.A.). Clustal X 1.81 (Thompson *et al.* 1997) was used to align the sequences, and then the alignment was refined by hand. The sequences were deposited in GenBank, and

aligned to a previously submitted alignment in TreeBase (SN 1244) (Chaverri *et al.* 2003a).

Analyses of molecular data

Neighbor-Joining (NJ) and Bayesian analyses were performed for selected sequences of each taxon. The outgroup taxa were *Nectria cinnabarina* and *Hypomyces stephanomatis*. The NJ trees were constructed using PAUP b8 version (Swofford 1999) using the Kimura 2-parameter model. MrBayes (Huelsenbeck 2000) was used to reconstruct phylogenetic trees using the Bayesian approach (Mau *et al.* 1999, Rannala & Yang 1996). MrBayes was run for 350,000 generations for each gene separately. Consensus trees were calculated using the 50% majority rule option in PAUP*. Bootstrap analyses were replicated 1000 times. A Shimodaira-Hasegawa (S–H) test (Shimodaira & Hasegawa 1999) was performed to test the significance of an alternative phylogenetic hypothesis; this analysis was done for each gene. The alternative hypothesis was a tree topology constructed by constraining species with green ascospores to be monophyletic. The likelihood of the resulting constrained tree was compared to that of the unconstrained. The models of DNA substitution were calculated using Modeltest 3.0 (Posada & Crandall 1998) and the results were the following: For RPB2 the Symmetrical model was used, with equal base frequencies, substitution model R(a) [A-C] = 1.2779, R(b) [A-G] = 4.4312, R(c) [A-T] = 1.0949, R(d) [C-G] = 0.6059, R(e) [C-T] = 7.6123, R(f) [G-T] = 1.000, proportion of invariable sites = 0.4203, and a gamma distribution shape parameter = 0.7863; and for EF-1α the Tamura-Nei model was used, with variable base frequencies (freqA = 0.2077, freqC = 03427, freqG = 0.2292, freqT = 0.2205), equal transversion frequencies, substitution model R(a) [A-C] = 1.0000, R(b) [A-G] = 2.2176, R(c) [A-T] = 1.000, R(d) [C-G] = 1.000, R(e) [C-T] = 6.9226, R(f) [G-T] = 1.000, proportion of invariable sites = 0.5754, and a gamma distribution shape parameter = 0.6746.

The Incongruence Length Difference Test or the Partition Homogeneity Test (PHT) in PAUP* was used to test the congruence among data sets (Cunningham 1997). For this test, parsimony-uninformative characters were excluded, gaps were treated as missing, and 500 repetitions were performed. A maximum of 100 trees was saved to conserve computer memory. RASA (Lyons-Weiler *et al.* 1996) Web Tool (http://bioinformatics.uml.edu/RASA.shtml) was used to detect potential problems with long-branch attraction (LBA). Taxa showing such effects were excluded from the analysis.

RESULTS

Molecular analyses

Table 4 shows the results of EF-1α and RPB2 sequence analyses. In general, RPB2 had more polymorphic sites than EF-1α, with 34% versus 21% for the latter. Most of the polymorphisms in RPB2 and EF-1α were found in the third codon position. The amount of homoplasy (*i.e.* parallel, convergent, reversed, or/and superimposed changes) found in the sequence data for both genes was high (*ca*. 0.7 homoplasy index) (Table 4). Whelan *et al.* (2001) stated that as homoplasy levels increase the likelihood of finding the correct evolutionary tree using Maximum Parsimony is progressively reduced. Therefore, we used NJ and Bayesian analyses.

Based on sequence analyses we distinguished several groups. Most species with green ascospores cluster in a large group, which we have designated GELATINOSA. Even if some hyaline-spored species are included, the grouping is not supported by bootstrap in the NJ EF-1α tree (Fig. 2). The GELATINOSA group is also not present in the EF-1α Bayesian tree (Fig. 3).

Table 4. Statistics of EF-1α and RPB2 sequence analyses.

Locus	EF-1α	RPB2	Combined
Total number of bp included	698	903	1601
Informative polymorphic sites (%)	143 (21)	304 (34)	443 (28)
Unique polymorphisms	58	66	110
Consistency index	0.32	0.33	0.32
Homoplasy index	0.68	0.67	0.68

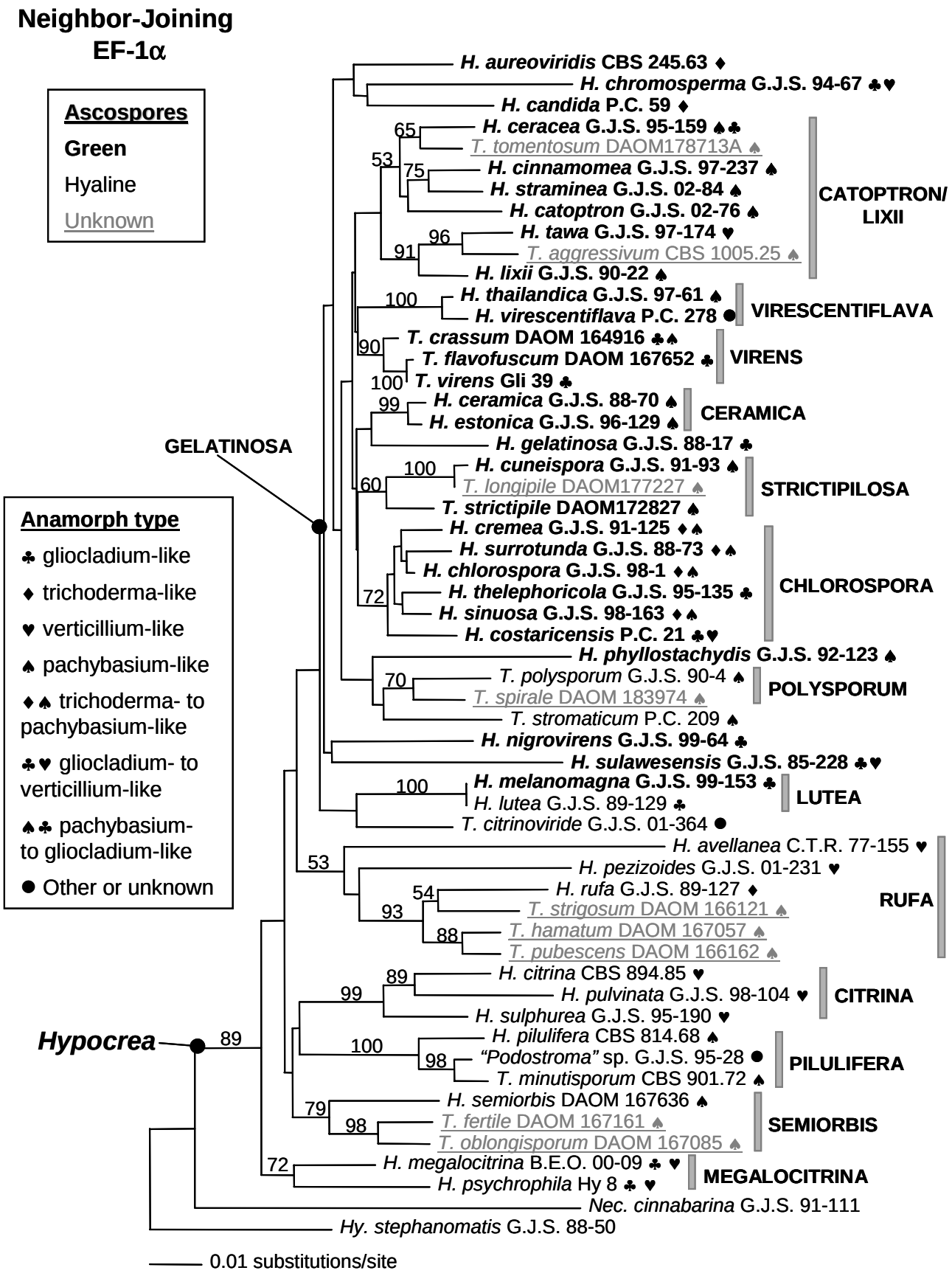

Fig. 2. EF-1α Neighbor-Joining tree. Bootstrap values > 50 % are shown at branches. Ascospore colour and conidiophore type are indicated.

Within this group, two species, *H. pachybasioides* (anamorph *T. polysporum*) and *Hypocrea stromatica* Bezerra *et al.* (anamorph *T. stromaticum*) have hyaline ascospores. The EF-1α NJ tree (Fig. 2) shows several subgroups with bootstrap values > 50% containing *Hypocrea/Trichoderma* species with green ascospores. We have designated these subgroups CATOPTRON, LIXII, VIRESCENTIFLAVA, VIRENS, CERAMICA, STRICTIPILOSA, CHLOROSPORA, and LUTEA. The LUTEA subgroup is the only one to include a species with hyaline ascospores, *H. lutea*. The same subgroups are also observed in the Bayesian analysis tree (Fig. 3), with the exception of VIRENS, the constituent elements of which, *T. crassum* and *T. virens/T. flavofuscum*, are seen as separate groups. In Fig. 2, CATOPTRON includes *H. ceracea/T. tomentosum, H. cinnamomea/H. straminea* groups, and *H. catoptron/T. catoptron*. LIXII includes *H. tawa/T. aggressivum* group, and *H. lixii*. VIRESCENTIFLAVA contains *H. thailandica/T. thailandicum* and *H. virescentiflava*. CERAMICA subgroup is formed by *H. ceramica/T. ceramicum* and *H. estonica/T. estonicum*. STRICTIPILOSA includes *H. cuneispora/T. longipile* group, and *H. strictipilosa/T. strictipile*. The close relationship of species in STRICTIPILOSA was also shown in Chaverri *et al.* (2003a). *Hypocrea cremea, H. surrotunda, H. chlorospora, H. thelephoricola*, and *H. sinuosa* comprise CHLOROSPORA. LUTEA includes *H. lutea* and *H. melanomagna*. The relationship of *Hypocrea gelatinosa*, the type of *Creopus* and *Chromocrea*, to other species is not supported in the NJ EF-1α tree. It is, however, closely related to STRICTIPILOSA in the EF-1α Bayesian tree (89% probability). The *H. spinulosa* sequence (isolate G.J.S. 99-25) evidenced a long-branch attraction when analyzed using the RASA tool. This sequence was therefore removed from the analyses.

The RPB2 NJ tree (Fig. 4) exhibits higher bootstrap support for terminal and some internal nodes than is seen in the other analyses. Most of the groups present in the EF-1α trees are also present in the RPB2 NJ and Bayesian trees (Figs. 4, 5). The only exception is the CATOPTRON subgroup, which is paraphyletic with LIXII nested within it. CATOPTRON and LIXII form a monophyletic group, hereafter designated CATOPTRON/LIXII, in the RPB2 trees. All other groups and subgroups of species of *Hypocrea/Trichoderma* with green ascospores are supported by bootstrap values > 80 %. The RPB2 trees show a close relationship of CHLOROSPORA to *H. aureoviridis*, *H. candida*, *H. spinulosa*, and the VIRESCENTIFLAVA subgroup. In addition RPB2 trees show that the GELATINOSA group containing most of the species with green ascospores is supported by a 93% bootstrap value in NJ (Fig. 4) and 99 % probability in the Bayesian tree (Fig. 5). In the Bayesian tree, *T. polysporum* is the only species in GELATINOSA that has a known teleomorph with hyaline ascospores. In the RPB2 trees, *H. gelatinosa* appears to be closely related to *H. chromosperma*.

The Partition Homogeneity Test (PHT) of the combined EF-1α and RPB2 sequences resulted in significant incongruence between data sets (p-value = 0.002). Most of the incongruences were encountered in the internal nodes; the combined NJ tree showed higher support for internal and terminal nodes than the individual data sets alone. Even though some authors have recommended not to combine partitions when the data sets are incongruent, we have combined the RPB2 and EF-1α sequence data following the precedents discussed in Cunningham (1997), Barker & Lutzoni (2002) and Chaverri *et al.* (2003a, b). Figure 6 shows a NJ tree with the combined sequence data. In Fig. 6 CHLOROSPORA, VIRESCENTIFLAVA, LIXII, VIRENS, CERAMICA, and STRICTIPILOSA subgroups are supported by bootstrap values higher than 95%. CATOPTRON, as in previous analyses of individual genes, is not monophyletic but it forms the monophyletic CATOPTRON/LIXII subgroup in combination with LIXII.

The individual and combined trees also show that anamorphic characters, such as type of anamorph and conidial colour are not monophyletic. Species with pachybasium-, gliocladium-, trichoderma-, or verticillium-like anamorphs do not form clades (Figs. 2–6). Most pachybasium-like anamorphs are in "Pachybasium A" and "Pachybasium B" (Kindermann *et al.* 1998); as was discussed in Chaverri *et al.* (2003a). Most species with hyaline conidia are in the basal groups such as CITRINA and PILULIFERA (Figs. 2–6).

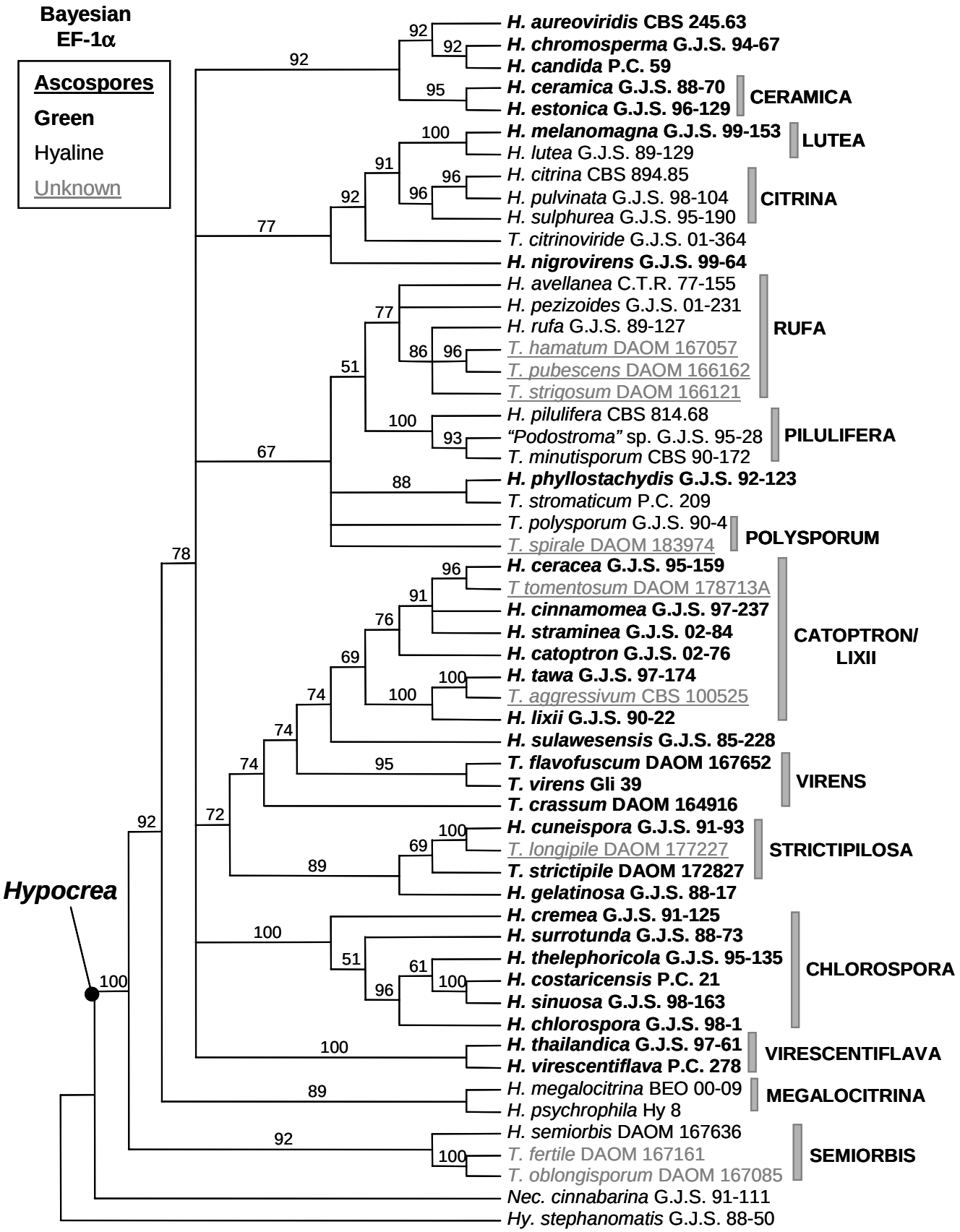

Fig. 3. EF-1α Bayesian tree. Probabilities (%) are shown at branches. Ascospore colour is indicated.

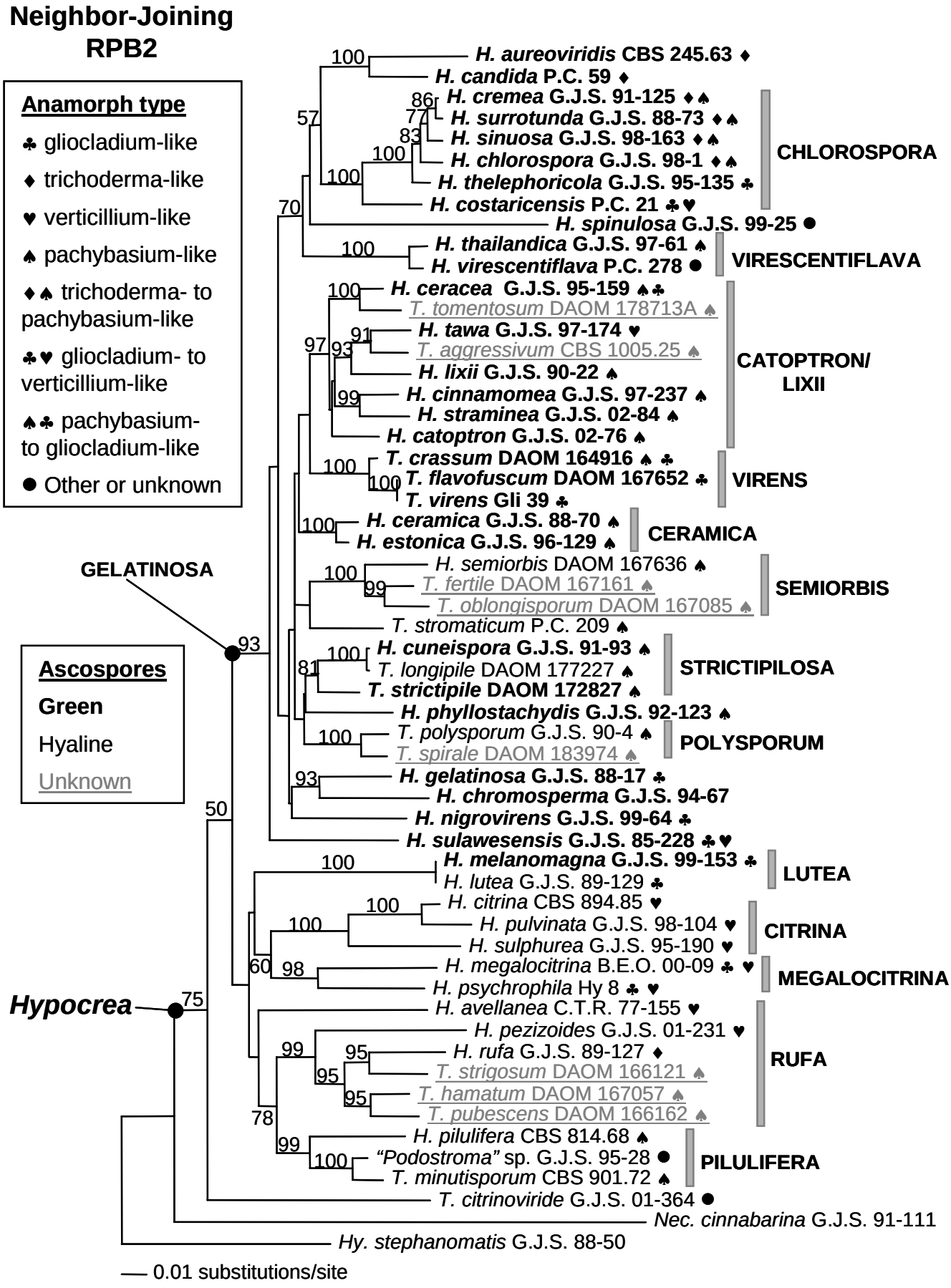

Fig. 4. RPB2 Neighbor-Joining tree. Bootstrap values > 50% are shown at branches. Ascospore colour and conidiophore type are indicated.

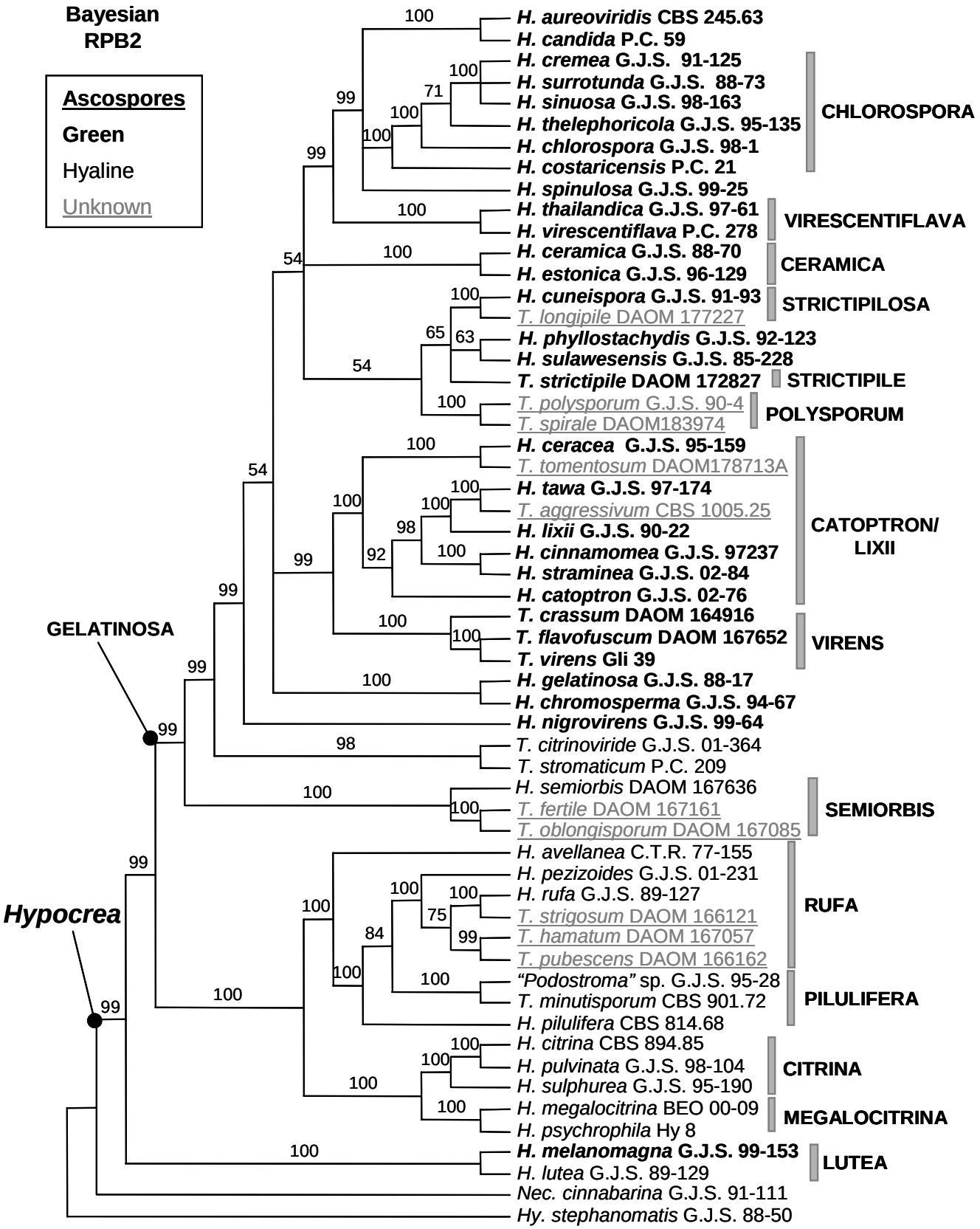

Fig. 5. RPB2 Bayesian tree. Probabilities (%) are shown at branches. Ascospore colour is indicated.

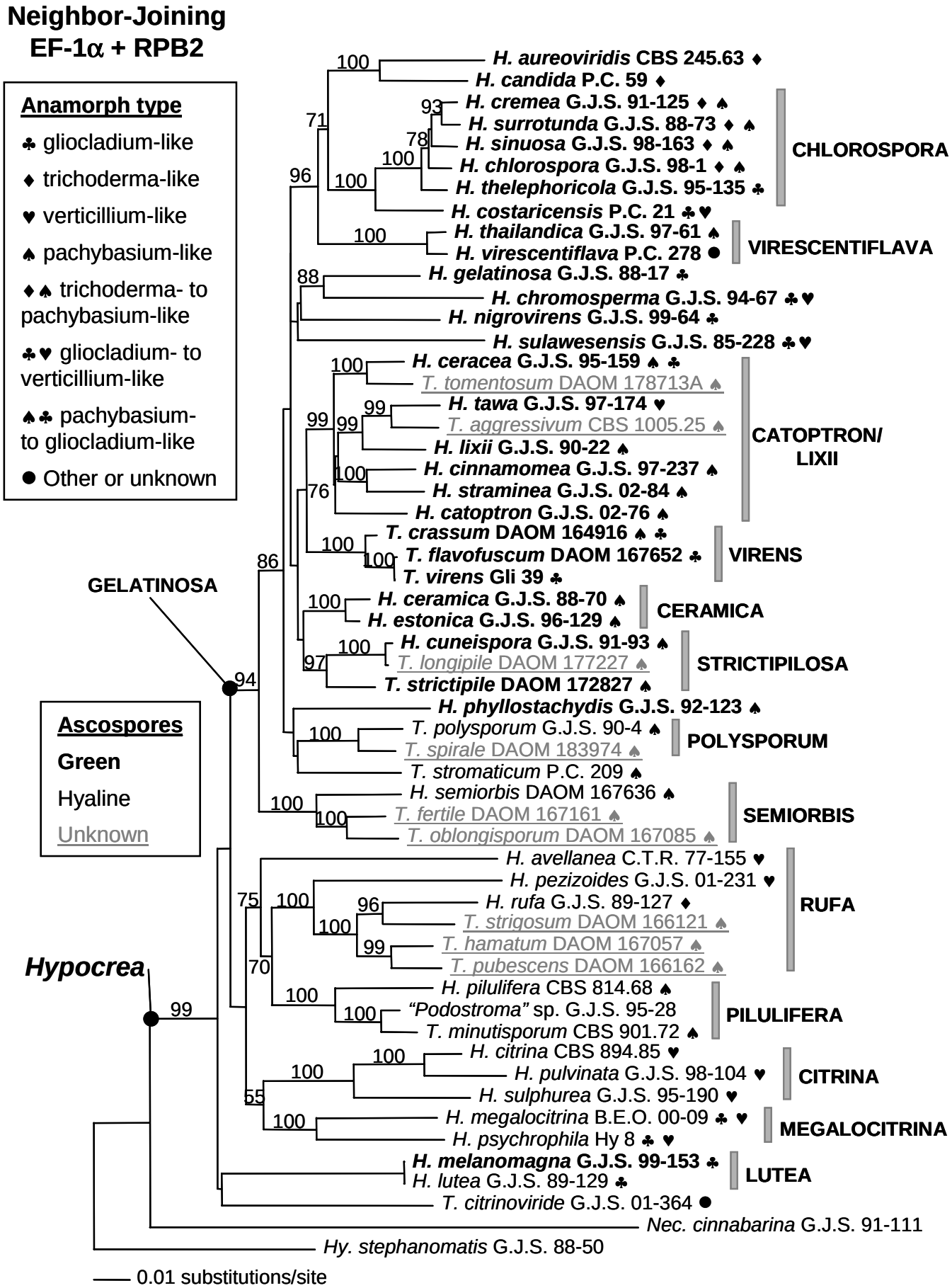

Fig. 6. Combined EF-1α and RPB2 Neighbor-Joining tree. Bootstrap values > 50% are shown at branches. Ascospore colour and conidiophore type are indicated.

Table 5. Shimoidara-Hasegawa test results [a].

Hypothesis	Locus	#Trees	Length	–ln *L* best	–ln *L* worst	p[b]
Unconstrained	EF-1α	7	800	4809.755	4813.431	
	RPB2	20	1866	9221.287	9233.213	
Green ascospores constrained	EF-1α	6	837	4914.980	4925.583	0.00[c]
	RPB2	40	1976	9494.127	9514.472	0.00[c]

[a] Taxa with unknown teleomorphs were excluded from the analyses. [b] Significantly less likely at p = 0.05, thus, null hypothesis that monophyly of the particular group is not an equally likely explanation of the data. [c] Indicates significantly worst explanations of the data.

As mentioned above, S–H analysis was performed to test the hypothesis that species with green ascospores formed a monophyletic group. RPB2 and EF-1α sequences, separately, of species with green ascospores were constrained into monophyly and phylogenetic trees were constructed that were then compared to the unconstrained trees. The results showed that the hypothesis of monophyly of species with green ascospores was not an equally likely explanation of the data when compared to the unconstrained trees, thus allowing rejection of the hypothesis of monophyly (Table 5).

CHLOROSPORA is a subgroup of phenotypically and genotypically closely related species (*i.e. H. cremea, H. surrotunda, H. chlorospora*, and *H. sinuosa*). Morphologically, the teleomorphs and anamorphs are almost indistinguishable. For these reasons, analyses of multiple isolates of species of CHLOROSPORA from different geographical origins were analyzed to distinguish among closely related species. Figure 7 shows three NJ gene trees of EF-1α, RPB2, and combined sequence data from several isolates of *H. chlorospora, H. costaricensis, H. cremea, H. sinuosa* and *H. surrotunda*. *Hypocrea candida, H. thailandica* and *H. aureoviridis* are included as outgroup species. In both RPB2 and EF-1α gene trees, isolates of *H. chlorospora, H. cremea, H. surrotunda*, and *H. sinuosa* each form monophyletic groups supported by bootstrap values > 75%. Morphological characters support the individual monophyletic species groups. *Hypocrea costaricensis* is basal to *H. cremea, H. surrotunda, H. sinuosa, H. chlorospora,* and *H. thelephoricola*. This relationship, however, is not observed in the large EF-1α trees (Figs. 2 and 3).

Morphological analyses of *Hypocrea* species with green ascospores

Stroma anatomy. The **stromatal size** of the species treated in this study ranges from 0.5–15.0 mm diam. Six out of the 40 species described (*i.e. H. albocornea, H. cornea, H. melanomagna, H. substipitata, H. sulawesensis,* and *H. velenovskyi*) have large stromata that range from 2–15 mm diam. The smallest stromata are those of *H. clusiae, H. estonica, H. nigrovirens,* and *H. thelephoricola*, which range between 0.4 and 0.7 mm in diam. The size of the stroma is generally correlated with the number of perithecia per stroma. The **colours of the stromata** of the species studied are in shades of white, yellow, orange, brown, green, or black. Yellowish colours are most common, being found in 22 species; these species do not form a monophyletic group (see Figs. 2–6). A few species, *i.e. H. cornea, H. lixii, H. melanomagna, H. nigrovirens, H. rugulosa,* and *H. tawa*, have dark brown or dark green to almost black stromata; however, only *H. lixii* and *H. tawa* are phylogenetically closely related to one another (see Figs. 2–6). The **shapes of the stromata** are somewhat conserved. Most stromata are pulvinate with a broad or narrow base, but a few are more or less disciform, peltate or almost cylindrical. *Hypocrea albocornea* has disciform stromata, *H. cornea* is somewhat peltate, and *H. substipitata* is short-cylindrical and seated on a thick, effused subiculum. The **surface of the stroma** can be shiny or dull/matt, opaque or semitransparent (waxy), glabrous or scurfy, and tomentose or roughened. Most species of *Hypocrea* have a stromatal surface that is glabrous and opaque with slight perithecial protuberances; however, there are many exceptions. For example, the stromata of *H. ceracea, H. chlorospora, H. cremea*, and *H. gelatinosa*, among others, have shiny, waxy, somewhat transparent stromata. *Hypocrea sulawesensis*, *H. macrospora,* and *H. substipitata* have a whitish tomentum on the surface of the stroma. *Hypocrea nigrovirens* and *H. tuberosa* are rendered conspicuously tuberculate by their perithecial protuberances. *Hypocrea rugulosa* is deeply furrowed or wrinkled. The reaction of stromatal tissue to 3% KOH is variable among species and it is not indicative of phylogenetic relationships at the infraspecific level. However, it can be useful at the species level in combination with other characters. Stromata of some species do not change in colour in KOH, while in others there is a weak or strong change. For example the stromata of *H. clusiae, H. cuneispora, H. gyrosa, H. straminea*, and *H. thailandica* change from yellowish to dark brown or reddish brown when KOH is added. Most other species either have no reaction or have a slight change to light brownish. The **tissue structure of the stroma surface** is highly conserved among species of *Hypocrea* with green ascospores.

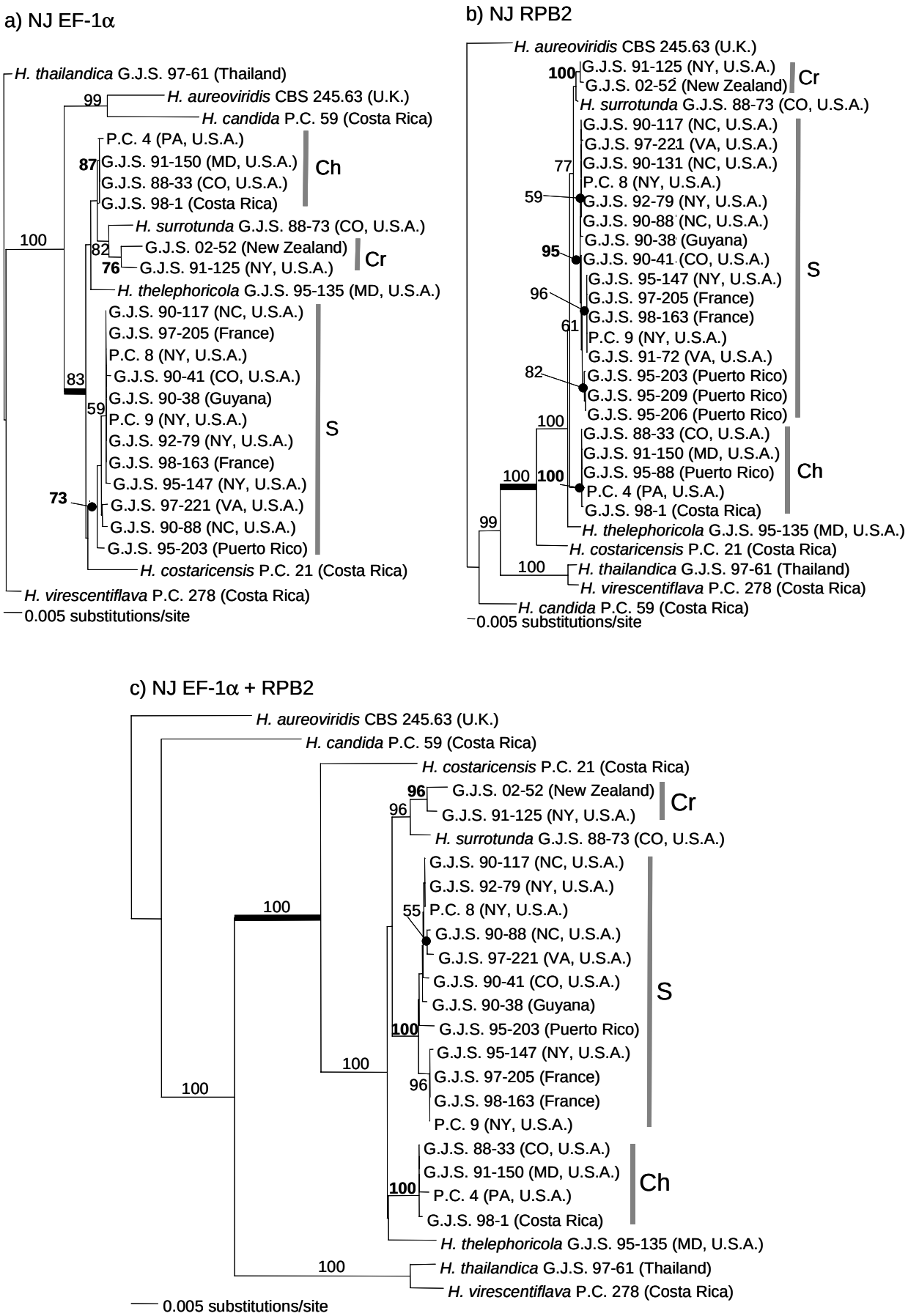

Fig. 7. Neighbor-Joining trees of species in the CHLOROSPORA group. a) EF-1α; b) RPB2; c) Combined EF-1α and RPB2. — *Hypocrea aureoviridis, H. candida, H. thailandica*, and *H. virescentiflava* are outgroup taxa. — Ch = *H. chlorospora* isolates; Cr = *H. cremea* isolates; S = *H. sinuosa* isolates. The thicker branch in each tree represents the CHLOROSPORA subgroup. Bootstrap values > 50% are shown at branches.

All species studied have the tissue type called *textura angularis*. Some differences are found in the thickness of the cell walls; for example, most species with dark-pigmented stromata, such as *H. lixii, H. tawa*, and *H. melanomagna*, have somewhat thickened cell walls. *Hypocrea cornea* has extremely thick cell walls (7.0–8.0 µm thick). Most other species have cell walls that range between *ca.* 0.5 and 1.5 µm thick. The **tissue below the outermost layer of the stroma** and between the perithecia is generally of *textura angularis, textura epidermoidea* or a condition intergrading between these tissue types. It is hyaline and possesses thin-walled cells. The stromatal **inner tissue below perithecia** is also generally of *textura angularis* to *textura epidermoidea* with thin-walled hyaline cells, though there are some exceptions. In *H. macrospora, H. melanomagna*, and *H. sulawesensis*, this layer is of *textura epidermoidea* intergrading with *textura intricata*. In this layer, *H. cornea* has extremely thick-walled cells 11.5–14.0 µm thick, making the stroma hard or coriaceous. Perithecia of *H. spinulosa* are partially immersed in a stroma formed by very loose interwoven hyphae.

Perithecium anatomy. Perithecia of *Hypocrea*, in longitudinal section, are generally elliptical to subglobose, sometimes globose, with walls *ca.* 10–20 µm thick, composed of 3–4 layers of highly compacted thin-walled cells. The walls of the perithecia of some species sometimes change colour from hyaline to light brownish when KOH is added.

Asci and ascospores. The **asci** of *Hypocrea* species with green ascospores are always cylindrical, with the tip thickened and with a inconspicuous ring. Ascospores are uniseriate. The length and width of the ascus is directly correlated to the size of the ascospores. The **part-ascospores** have a conserved morphology, in the vast majority of species being spinulose to warted, generally dimorphic, and 4–6 µm diam. **Ascospores** of some species, such as *H. macrospora* and *H. tuberosa*, have large warts. Others, such as *H. melanomagna*, have small warts and are almost smooth. Most part-ascospores are dimorphic, the distal part being generally globose to subglobose and the proximal part wedge-shaped, ellipsoidal, or subcylindrical. A few species have monomorphic globose part-ascospores. *Hypocrea aureoviridis, H. candida, H. chlorospora, H. macrospora, H. sinuosa, H. virens, H. virescentiflava* have this character. Most of these species are phylogenetically closely related (Figs. 2–7), with the exception of *H. macrospora* (molecular data not available) and *H. virens*. The colour of the part-ascospores does not vary greatly. For the most part, the species studied have part-ascospores *ca.* 4–6 µm diam; this is true of 68% of the species studied. *Hypocrea aureoviridis, H. candida, H. clusiae, H. cornea, H. phyllostachydis, H. sulawesensis*, and *H. thelephoricola* have small part-ascospores 2.5–4.0 µm in diam. *Hypocrea albocornea, H. centristerilis*, *H. cuneispora, H. velenovskyi,* and *H. virescentiflava*, have large part-ascospores 6.5–8.5 µm in diam. *Hypocrea macrospora* and *H. tuberosa* have extremely large part-ascospores 10.0–13.5 µm in diam.

A high degree of variation was encountered in the morphology of **anamorphs** of the species studied. This finding was in contrast to the conserved morphology of the corresponding teleomorphs. Anamorph morphology was useful in distinguishing species, although, in many cases, there was variation within one species (*e.g. H. lixii/T. harzianum*).

Colony. The extent of variation in appearance of colonies on CMD at 20 ºC after 1–2 week of growth is significant at the species level. The majority of the species studied grow as flat colonies with moderately to highly aggregated conidiophores, forming compact to loose pustules or tufts. Some species form a verticillium- or gliocladium-like mononematous synanamorph in greater or lesser abundance in the aerial mycelium along with the pustulate anamorph (see also Chaverri *et al.* 2003a). Species with this character include *H. catoptron, H. cinnamomea, H. crassa, H. cremea, H. cuneispora, H. estonica, H. surrotunda, H. strictipilosa,* and *H. straminea*. In less common cases, the colonies form only verticillium- or gliocladium-like conidiophores, which may be scarce or abundant. This character is typical of *H. costaricensis, H. gelatinosa, H. melanomagna, H. virens, H. nigrovirens, H. chromosperma, H. tawa,* and *H. thelephoricola*. Colonies on **PDA at 25 ºC** after 1–2 weeks of growth are also highly variable. Pustules are generally not observed when isolates are grown on PDA, though *H. aureoviridis, H. ceramica, H. chlorospora, H. phyllostachydis, H. sinuosa*, and *H. straminea* are exceptions to this generality. No distinctive **odour** was noticed in any of the species studied. Production of **diffusing pigment** was not commonly observed, but when such a pigment was present, it was often inconsistently formed within individual species. *Hypocrea aureoviridis, H. ceracea, H. costaricensis*, *H. lixii,* and *H. virens* produced yellow to brownish diffusing pigments. *Hypocrea aureoviridis* produced yellow granular inclusions in the hyphae on malt extract agar. The highest level of variability was found within *H. lixii/T. harzianum*, which produced pigments ranging from pale yellowish, to brownish or reddish brown.

Conidiophores. Conidiophore morphology is variable, sometimes even within individual species. The majority of the *Trichoderma* anamorphs of *Hypocrea* species with green ascospores have pachybasium-like morphology with irregular branching patterns. The pachybasium-like conidiophore typically includes an

identifiable main axis; often the main axis terminates in a sterile or terminally fertile extension. Fertile branches arise near the base of the extension or along the length of the main axis in cases when no extensions are formed. The branches tend to be paired or in whorls, but unilateral branching is also common. The fertile branches often rebranch and the secondary branches are paired or unpaired. Branches often are not uniformly spaced and the archetypical 'pyramidal' construction that is central to the traditional form-concept of *Trichoderma* is not apparent. Each branch terminates in one, a few, or numerous phialides. Sometimes the phialides are in whorls of 3–4 with each phialide disposed at or near a 90° angle to its nearest neighbours. Other species have more typical trichoderma-like conidiophores. These also branch irregularly, generally once. Branches are attached at wide angles, and are paired or single, with 2–3 phialides per whorl. This type of structure is found, for example, in *H. aureoviridis, H. candida,* and *H. cremea*. In some cases, the conidiophores have a morphology that is intermediate between trichoderma- and pachybasium-like. Examples are *H. chlorospora, H. sinuosa,* and *H. thailandica*. Verticillium- and gliocladium-like conidiophores are less common than pustulate conidiophores. *Hypocrea costaricensis* and *H. tawa* have verticillium-like conidiophores; and *H. crassa*, *H. gelatinosa, H. melanomagna, H. nigrovirens, H. thelephoricola*, and *H. virens* have gliocladium-like conidiophores. Verticillium-like anamorphs are characterized by mononematous conidiophores with few branches that arise at relatively narrow angles to the conidiophore axis. There are 1–3 phialides per metula. Phialides are slender, and conidia are green. Gliocladium-like conidiophores have branches arising at narrow angles, bending towards the tip of the conidiophore. Phialides are crowded and arise at narrow angles, giving rise to wet, slimy masses of green conidia. Mononematous anamorphs can be formed on the agar or on aerial hyphae. Some pustule-forming species produce **synanamorphs** that are verticillium-like and that are formed in the aerial hyphae or within the pustule. This character is found in *H. catoptron, H. cinnamomea, H. cremea, H. cuneispora, H. estonica, H. straminea, H. strictipilosa, H. surrotunda,* and *H. thailandica*. Synanamorphs may also be gliocladium-like, as in *H. crassa*.

Phialides. Phialides are generally ampulliform or lageniform, straight but sometimes hooked, as seen in *H. chlorospora, H. cinnamomea, H. costaricensis, H. sinuosa,* and *H. thailandica*. Rarely, they are twisted, as is seen in *H. chromosperma*. Phialides of some species are longer and more slender than is typical; however, such phialides are usually found only in the verticillium- or gliocladium-like anamorphs. Phialides of most species measure 5.5–8.0 × 3–4 µm. Phialides of *Hypocrea aureoviridis, H. candida, H. cinnamomea, H. cremea, H. gelatinosa, H. melanomagna, H. nigrovirens, H. sulawesensis, H. tawa, H. thelephoricola*, and *H. virens* measure 8.0–15 × 3–4 µm. *Hypocrea costaricensis* and *H. estonica* have extremely long (*ca.* 15–25 µm) and *H. straminea* has very short phialides (4.7–5.0 µm). Short and wide phialides have an L/W of 1.5–2.0, as in *H. catoptron, H. clusiae, H. cuneispora, H. strictipilosa*, and *H. straminea*; long and slender phialides have a L/W of 4.5–8.0, as in *H. aureoviridis, H. costaricensis, H. estonica, H. melanomagna, H. tawa*, and *H. thelephoricola*.

Conidia. The conidia of the species of *Hypocrea/Trichoderma* studied here are green and smooth, except in the case of *T. flavofuscum*, which has yellowish conidia. Conidia of most species are 3.0–4.5 × 3.0–4.0 µm, but in some species they are smaller or larger. *Hypocrea costaricensis, H. cuneispora, H. sinuosa, H. strictipilosa, H. surrotunda, H. tawa*, and *H. virens* have 4.5–6.0 µm long conidia and in *H. nigrovirens* they are 6.0–6.5 µm long. *Hypocrea catoptron, H. ceramica, H. clusiae, H. lixii, H. melanomagna, H. phyllostachydis, H. straminea, H. sulawesensis*, and *H. thelephoricola* have 1.5–3.0 µm wide conidia. Most of the species have subglobose to ellipsoidal conidia (L/W 1.2–1.4); some species, viz. *H. candida, H. chlorospora, H. cremea, H. lixii*, and *H. thailandica*, have almost globose conidia (L/W 1.1–1.2) while other species, viz. *H. catoptron, H. costaricensis, H. cuneispora, H. chromosperma, H. straminea, H. tawa*, and *H. thelephoricola*, have oblong conidia (L/W 1.4–1.8).

Chlamydospores. Observed in some species and isolates. In *H. costaricensis, H. cuneispora, H. lixii, H. sinuosa*, and *H. virens*. *Hypocrea costaricensis, H. cuneispora*, and *H. virens* chlamydospores are abundant in the aerial and the submerged mycelium in young and old colonies. When they occur, are generally globose to subglobose, thick-walled, hyaline, and terminal or intercalary. Chlamydospores of *Hypocrea costaricensis* sometimes exhibit a faint brownish colouration.

Growth rate. There is a wide range of growth rates among the species studied. The optimum for growth of all species on PDA and SNA is between 25 ºC and 30 ºC and few grow at 35 ºC. The rate of growth can be useful in distinguishing species. For example, *T. aggressivum* and *T. harzianum* (teleomorph *H. lixii*) can be distinguished by the ability of *T. harzianum* to grow at 35 ºC. On the other hand, *T. virens* (teleomorph *H. virens*) and *T. flavofuscum* have indistinguishable growth curves, as previously shown by Chaverri *et al.* (2003a: Fig. 1). Some species such as *H. costaricensis, H. cremea, H. straminea*, and *H.*

strictipilosa show moderate growth at 35 ºC, giving rise to colonies 2–10 mm in radius after 3 d. Other species, namely, *H. lixii, H. melanomagna, H. nigrovirens*, and *H. virens*, grow rapidly at 35 ºC, giving rise to colonies 10–40 mm radius after 3 d. Although in most species there is little difference in the growth rates seen at 25 and 30 ºC, *H. aureoviridis* and *H. estonica* have a sharp drop in growth rate from 25 to 30 ºC. The growth rate on PDA is generally faster than that seen on SNA, except in *H. virens/T. virens* and *T. flavofuscum* (see also Chaverri *et al.* 2003a). In general, *H. aureoviridis, H. cinnamomea, H. costaricensis*, and *H. thelephoricola* have slow growth, with colony radii at 25 ºC after 3 d < 20 mm. *Hypocrea catoptron*, *H. chlorospora, H. ceramica, H. cremea, H. cuneispora, H. lixii, H. melanomagna, H. nigrovirens, H. sinuosa, H. straminea, H. strictipilosa,* and *H. virens* grow rapidly, with colony radii at 25 ºC after 3 d > 35 mm.

Species in the CHLOROSPORA subgroup are similar in teleomorphic and anamorphic morphology. Table 6 shows a summary of the diagnostic teleomorph and anamorph features of the CHLOROSPORA subgroup. A detailed description of each species is given in the taxonomy section. Significant differences in colony appearance and growth rates among species in the CHLOROSPORA subgroup were found; these are shown in Table 6 and Fig. 8. Figure 8 shows the growth curves of species in the CHLOROSPORA group. *Hypocrea chlorospora, H. cremea*, and *H. sinuosa* all have rapid growth.

Substrata. Hypocrea species with green ascospores form teleomorphic structures on decaying woody substrata (with bark or decorticated), on ascomycetes or basidiomycetes, and rarely on leaves or on herbaceous monocotyledonous plants. *Hypocrea atrogelatinosa, H. catoptron, H. ceramica, H. estonica, H. lixii, H. strictipilosa*, and *H. thelephoricola* grow on basidiomycetes (*Aphyllophorales s. l.*); none have been found on basidiomata of *Agaricales s. l. Hypocrea chromosperma* and *H. strictipilosa* grow on black pyrenomycetes. *Hypocrea clusiae* was found on leaves of *Clusia* sp. *H. phyllostachydis* grows on decaying culms of the bamboo *Phyllostachys bambusoidea*, *H. virescentiflava* was described from culms of an unidentified bamboo, and *H. spinulosa* was described from alpine/boreal grasses. The rest of the species studied grow on bark or decorticated wood. In the majority of the cases, the substrata we examined were blackened, indicating the possibility that these *Hypocrea* species were growing on dematiaceous mycelia of unidentified ascomycetes. In other cases longitudinal sections of the stroma revealed that it was seated on dematiaceous mycelia. *Hypocrea chlorospora, H. cremea*, and *H. sinuosa* grow on well-rotten, often wet decorticated wood. The well-rotten wood resembled wood affected by soft-rot, which is sometimes caused by *Agaricales s. l.*

DISCUSSION

Generic concept and the GELATINOSA group

As mentioned above, *Creopus* and *Chromocrea*, both based on *H. gelatinosa*, have been segregated from *Hypocrea* because of their formation of green ascospores. Based on common anamorph and teleomorph characters, this distinction has been generally abandoned in recent decades. Preliminary DNA and morphological evidence suggested that a segregation of *Creopus* and *Chromocrea* is not justified (Chaverri *et al.* 1999). In the present study, based on RPB2 and EF-1α gene genealogies, it could conclusively be demonstrated that all *Hypocrea* species with green ascospores were derived from within the genus *Hypocrea*. The Shimoidara-Hasegawa test proved that species with green ascospores should not be segregated from *Hypocrea*. It was also found that most species with green ascospores, except *H. melanomagna*, were in a derived group (GELATINOSA), which is supported by high bootstrap values in the RPB2 and combined RPB2 and EF-1α genealogies (Figs. 2–6). However, the limits of GELATINOSA are not well resolved because the position of SEMIORBIS is unclear. EF-1α trees show that SEMIORBIS is outside GELATINOSA, while RPB2 trees and EF-1α/RPB2 combined, show SEMIORBIS nested within GELATINOSA. *Hypocrea melanomagna* occupies a position that is basal yet outside the GELATINOSA group. In this group, no teleomorphic characters other than ascospore colour are significantly divergent from the typical concept of *Hypocrea*. In addition, the anamorphs of *Hypocrea* species with green ascospores fit the concept of *Trichoderma*, as is the case with the anamorphs of other *Hypocrea* species. Therefore, we conclude that *Creopus* and *Chromocrea* are synonyms of *Hypocrea*.

Evolution of teleomorph characters

Most species in the *Hypocreales* have hyaline ascospores. Genera that are probably ancestral to *Hypocrea* (*e.g. Arachnocrea, Aphysiostroma, Hypomyces, Sphaerostilbella*) also have hyaline ascospores. Figures 2–6 show that the majority of the basal subgroups of *Hypocrea* (MEGALOCITRINA, SEMIORBIS, PILULIFERA, CITRINA, and RUFA) have hyaline ascospores, except *H. melanomagna*, which groups with *H. lutea* in a basal position; the more derived species composing the GELATINOSA group have green ascospores. Kullnig-Gradinger *et al.* (2002) presented a multi-gene study of the phylogeny of *Trichoderma*. They included mostly species with teleomorphs producing hyaline ascospores. Species with

green ascospores included in their study were *T. virens, H. tawa, T. harzianum, T. strictipile*, and *H. aureoviridis*. In their phylogenetic trees, species with green ascospores show significantly different phylogenetic relationships to species with hyaline ascospores than those presented in our study. However, these results could have been affected by the inappropriate selection of *H. aureoviridis* as outgroup. With the inclusion of additional closely related species, *H. aureoviridis* is seen to be derived rather than basal, and closely related to the CHLOROSPORA subgroup (Figs. 2–6).

Stromatal anatomy is highly conserved in *Hypocrea*. The stroma of most species is pulvinate to discoidal and has a differentiated, pigmented surface region that is underlain by somewhat loosely intertwined hyphae or pseudoparenchymatous tissue. The anatomy of the surface region is more or less variable according to groups of species. There is a tendency for stromata of more basal species to be effused, sometimes indefinite in extent, and thus suggestive of genera outside of *Hypocrea* such as *Hypomyces* and *Aphysiostroma.* However other species that are also basal, such as *H. semiorbis*, *H. lutea* and *H. rufa,* have pulvinate to discoidal stromata. Interestingly, the immature stroma of *H. rufa* and its relatives is semi-effused when young, most often becoming pulvinate as it matures. Other examples of this kind of stroma are in the CITRINA subgroup, *H. megalocitrina*, and *H. psychrophila* (Canham 1969, Doi 1972, Müller *et al.* 1972, respectively). However, subgroups such as SEMIORBIS and LUTEA, which are also basal, have pulvinate stromata composed of tissue types of *textura angularis* to *textura epidermoidea*. *Hypocrea spinulosa* has a stroma formed of very loose interwoven hyphae. Its relationship to other species with similar teleomorphic structures cannot yet be clarified because in the EF-1α trees its sequence presented a long-branch attraction. RPB2 and EF-1α sequencing shows that the anatomy of the stroma is a polyphyletic character. Other teleomorphic characters, such as specific KOH reactions, stromatal surface types, stromatal size features, and stromatal colours, have also arisen and been lost multiple times in the evolution of *Hypocrea/Trichoderma*. These characters are not useful to recognize groups at the infraspecific level, but are, in many cases, useful to distinguish individual species. In conclusion, similarity in teleomorphic characters is not indicative of close phylogenetic relationships.

Doi (1972) developed a classification system for *Hypocrea* based mainly on characteristics of the teleomorph; more specifically, stromatal tissue microanatomy was emphasized. Based on the results of the present study, we conclude that Doi's *H.* subsection *Creopus* is an artificial group. Doi included all species with green ascospores in this group and a few species with hyaline ascospores, such as *H. lutea*. We found that species with green ascospores do not form a monophyletic group and that *H. lutea* is a basal species, not closely related to *H. gelatinosa,* the type species of *H*. subsect. *Creopus* (Link) Yoshim. Doi.

Doi also used stromatal tissue characteristics to segregate subsect. *Creopus*. He stated that members of this group had *textura angularis* surface tissue composed of cells that generally were thick-walled and pigmented. We found that other species that are not in subsect. *Creopus*, such as *H. pachybasioides*, also have stromata composed of *textura angularis* tissue. In addition, several of the species with green ascospores, *e.g. H. chlorospora, H. cremea,* and *H. costaricensis*, lack pigmented or thick-walled stromatal surface cells.

Evolution of anamorph characters

Most anamorphs in *Hypocrea* are readily recognizable as being *Trichoderma*. The *Trichoderma* anamorph is unique in the ascomycetes while verticillium- and gliocladium-like morphologies (more specifically the characteristic conidiophore branching patterns seen in these morphs) have occured many times in the ascomycetes. These relatively simple anamorphs are found in the more basal groups of *Hypocrea*, as well as in genera such as *Hypomyces s. l.*, *Arachnocrea*, and *Aphysiostroma* that are hypothetically ancestral to *Hypocrea s. l.* A higher degree of complexity in conidiophore branching is seen in more derived groups; however, there are exceptions.

For example, the basal groups RUFA and PILULIFERA have complex trichoderma- and pachybasium-like anamorphs. The most basally situated subgroups, that is, MEGALOCITRINA, LUTEA, and CITRINA, have acremonium-, gliocladium- or verticillium-like conidiophores. Anamorphs of hypothetically ancestral and closely related genera, viz. *Aphysiostroma, Arachnocrea, Sporophagomyces*, and *Sphaerostilbella*, also have gliocladium- or verticillium-like conidiophores. On the other hand, the more derived species comprised within the GELATINOSA group have more "complex," pachybasium- or trichoderma-like conidiophore morphology. RPB2 and EF-1α gene phylogenies suggest that "simpler" conidiophore morphology, as seen in gliocladium- and verticillium-like conidiophores, is probably an ancestral character, and that more "complex" conidiophore morphology is derived from within *Hypocrea/Trichoderma*. In addition, conidia produced from gliocladium- and verticillium-like conidiophores are held in wet, slimy heads, while conidia formed from pachybasium- and trichoderma-like conidiophores usually have dry conidial masses. Conidial masses of some isolates of *T. harzianum,* which is pachybasium-like, may appear to be slimy.

Conidial colour was found to be a character that has converged multiple times in *Hypocrea/Trichoderma*. The majority of species have

green conidia; however, a few species, such as *T. polysporum* and members of the CITRINA, PILULIFERA, and MEGALOCITRINA subgroups, have colourless conidia. Species with colourless conidia are found in the basal subgroups, as well as in most other genera in the *Hypocreales*, while species with green conidia are derived from within *Hypocrea/Trichoderma*. Species with green conidia are also found in certain basal subgroups such as RUFA and LUTEA. None of the anamorphs of *Hypocrea* species with green ascospores has hyaline conidia. Obviously, the production of green melanin-type pigments is a widespread capacity in *Hypocrea*, that mostly is activated in the conidia, sometimes also in the ascospores.

Subgroups of species with green ascospores

There are several subgroups that contain mostly species with green ascospores. They are: CATOPTRON/LIXII, VIRESCENTIFLAVA, VIRENS, CERAMICA, STRICTIPILOSA, CHLOROSPORA, and LUTEA. Of these subgroups, LUTEA is the only one that contains a species with hyaline ascospores, namely, *H. lutea*. Groups of species with hyaline ascospores fall outside of the scope of this study and are not discussed in detail. However, as mentioned before, most species/groups with hyaline ascospores are in the basal groups, as seen in Figs. 2–6.

Hypocrea aureoviridis/H. candida subgroup. Hypocrea aureoviridis and *H. candida* form a monophyletic group in the RPB2 and combined trees (Figs. 4–6) supported by bootstrap values of 100%. These two species are also morphologically very similar. The anamorphs have trichoderma-like conidiophores with simple branching and few phialides. The conidia are green, small and almost globose; the ascospores are green and globose to subglobose, with nearly smooth surfaces ornamented with very small warts. Differences between the species can be found in the colour of the stromata – yellowish in *H. candida* and somewhat orange-brown in *H. aureoviridis* – as well as in the sizes of the phialides and conidia, the tendency to form pustules or not on CMD and PDA, and the geographic distribution of the species. (*H. aureoviridis* occurs in Western Europe and *H. candida* in Costa Rica.)

CATOPTRON/LIXII subgroup. The CATOPTRON/LIXII subgroup includes *H. ceracea, T. tomentosum*, *H. cinnamomea, H. straminea, H. catoptron, H. tawa, H. lixii*, and *T. aggressivum*. This group is monophyletic in the RPB2, EF-1α and combined trees (Figs. 2–6) but it is weakly supported in the EF-1α trees. Most of the teleomorphic species in this group have darkly pigmented stromata, except *H. straminea* and *H. catoptron* in which stromata are greyish yellow. In addition, species in this group have pachybasium-like anamorphs and green ascospores with the exception of *H. tawa*, which has a verticillium-like anamorph. In *T. harzianum* and *T. aggressivum* conidiophore morphology is intermediate between pachybasium- and trichoderma-like. Species in this group are also fungicolous, except *T. tomentosum* of which the original description mentions the substratum *Ulmus* sp. bark (Bissett 1991b). As detailed in the Introduction above, *Hypocrea lixii/T. harzianum* is an important biocontrol agent of fungal plant pathogens and *T. aggressivum* is the agent that causes the "green mold" disease of the mycelium of commercially grown mushrooms.

VIRESCENTIFLAVA subgroup. The VIRESCENTIFLAVA subgroup consists of *H. virescentiflava* and *H. thailandica*. The close relationship between these two species is strongly supported in the RPB2 and EF-1α trees. However, no significant morphological characters were found to be consistent in both species. *Hypocrea virescentiflava* grows on bamboo and *H. thailandica* on decorticated wood.

CHLOROSPORA subgroup. The CHLOROSPORA subgroup consists of *H. cremea, H. surrotunda, H. chlorospora s. str.,* and *H. sinuosa*; these species are morphologically almost indistinguishable in their teleomorphs. This group of species was hypothesized to be a species complex. All members have pale yellow, semi-translucent stromata, and globose to subglobose, green ascospores. The anamorphs of *H. sinuosa* and *H. chlorospora* are very similar to one another, but small differences can be found in sizes of conidia, phialides and ascospores, as well as in growth rate (Table 6, Fig. 8). *Hypocrea chlorospora, H. cremea, H. surrotunda*, and *H. sinuosa* grow on well-rotten, decorticated wood, while *H. thelephoricola* grows on the hymenium of a member of the *Thelephoraceae*. It is possible that the species recorded from wood could also be fungicolous because mycelium of other fungi can be observed in longitudinal sections of the stromata of some species.

CERAMICA subgroup. The CERAMICA subgroup consists of *H. ceramica* and *H. estonica*. Chaverri *et al.* (2003a) mentioned that these two species were similar in the small size of their conidia. Both species possess pachybasium-like anamorphs with conidiophore elongations. The teleomorphs are very distinct: the stroma of *H. ceramica* is reddish brown and that of *H. estonica* is greyish yellow.

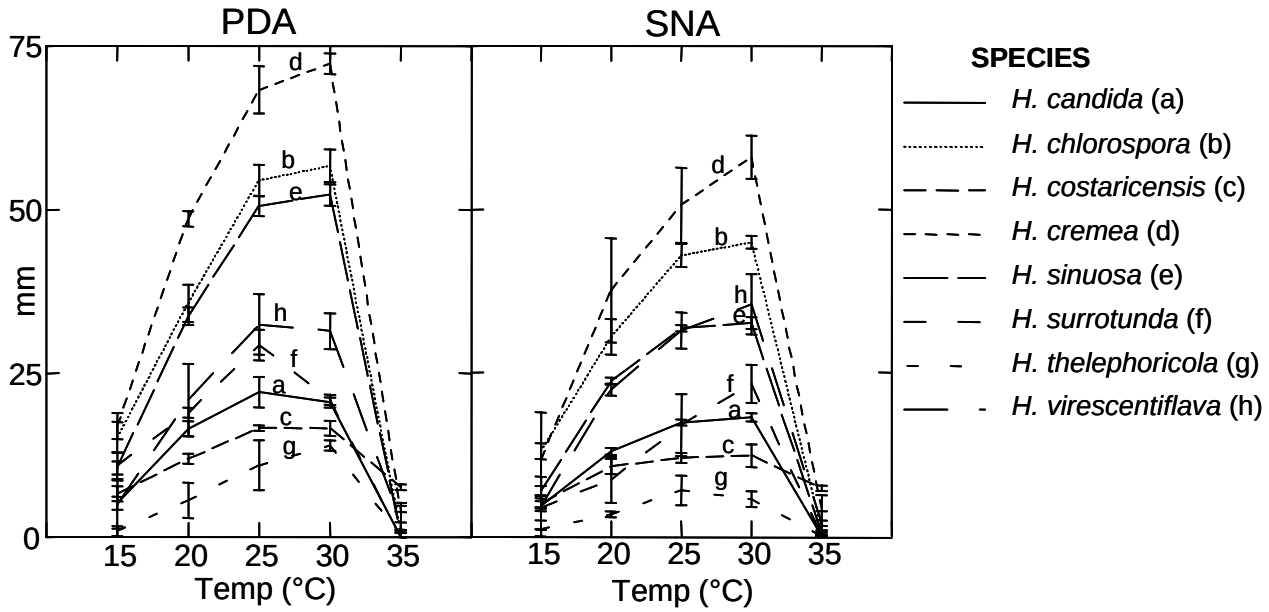

Fig. 8. Colony radii after 3 d on PDA and SNA at 15, 20, 25, 30, and 35 ºC, of species in the CHLOROSPORA subgroup.

STRICTIPILOSA subgroup. This subgroup is formed by *H. strictipilosa/T. strictipile*, *H. cuneispora* and *T. longipile*. The phylogenetic relationship of these species was already shown in Chaverri *et al.* (2003a). *Hypocrea strictipilosa* and *H. cuneispora* have relatively large green ascospores and stromata in shades of yellow. The anamorphs of the three species are pachybasium-like, with ellipsoidal to oblong green conidia and with conidiophore elongations that are generally fertile. Chaverri *et al.* (2003a) also demonstrated that *T. fasciculatum* is a synonym of *T. strictipile*.

VIRENS subgroup. The VIRENS subgroup includes *H. virens/T. virens* (= *T. flavofuscum*) and *H. crassa/T. crassum*. The general characteristics of this group are fast growth rate, gliocladium-like anamorphs, and yellowish stromata. *Hypocrea virens/T. virens* is distinguished from *H. crassa/T. crassum* by its faster growth at 25–30 ºC, as well as by its ability to grow at a moderate rate at 35 ºC and by its abundant chlamydospore production.

Trichoderma flavofuscum has the same growth characteristics as *H. virens/T. virens*, including production of abundant chlamydospores and of a gliocladium-like anamorph. It was originally distinguished from *T. virens* by its yellowish conidia. *Trichoderma virens* and *T. flavofuscum* are the only species or isolates of *Trichoderma* known to produce the mycotoxins gliotoxin and viridin (Brian & Hemming 1945, Brian & McGowan 1945, Webster 1964, Avent *et al.* 1993). In addition, the isolates of *H. virens/T. virens* and *T. flavofuscum* grow faster on SNA than on PDA, grow moderately fast at 35 ºC, and have identical RPB2 and EF-1α sequences (see Chaverri *et al.* 2003a, Fig. 1). Therefore, *T. flavofuscum* is considered a synonym of *T. virens*

LUTEA subgroup. The anamorph of *H. melanomagna* is almost indistinguishable from *Gliocladium viride* (teleomorph *H. lutea*). In addition, RPB2 and EF-1α sequences are almost identical. The readily perceptible difference between the two species lies in the teleomorph. *Hypocrea lutea* has small, pale yellow stromata and hyaline ascospores, while *H. melanomagna* has large, dark brown stromata and green ascospores. It is possible that *H. melanomagna* is a form of *H. lutea*; however, more specimens and cultures of *H. melanomagna* are needed to test this hypothesis. Therefore, in this paper, the new species *H. melanomagna* is retained as separate.

Ecology

Hypocrea lixii/T. harzianum and *H. virens/T. virens* are the only species with green ascospores that have clearly proven antifungal activities useable in biocontrol. However, because many species are fungicolous, there is potential for some of these species also to be effective against phytopathogens. In particular, species other than *H. lixii/T. harzianum* in the CATOPTRON/LIXII subgroup and other than *H. virens/T. virens* in the VIRENS subgroup would seem to be good candidates for further study. These two subgroups are phylogenetically related (Figs. 2–6). The close phylogenetic relationship of *T. virens* and *T. harzianum* was also discussed in Samuels *et al.* (2000). *Trichoderma stromaticum*, a species with a teleomorph producing hyaline ascospores, viz. *Hypocrea stromatica* (Bezerra *et al.* 2003), is nested within the GELATINOSA group; it has biocontrol activities against cacao witches' broom caused by

Crinipellis perniciosa (Samuels *et al.* 2000). It is, in fact, a direct parasite of *C. perniciosa* mycelium (J. de Sousa, pers. comm.)

Most species of *Trichoderma* disperse readily in water, but there is a tendency for conidia of species that form pustules, such as *H. lixii/T. harzianum*, *H. chlorospora* and *H. phyllostachydis*, not to possess this capability. The formation of pustulate conidiophores is often associated with the formation of protruding, sterile hairs or conidiophores. These could be adaptations to dispersal by animals such as arthropods or nematodes. It was also observed that the pustules are often easily dislodged from the agar surface, so that the entire pustule/conidiophore could be dispersed as a unit.

The conidia produced in slimy masses on gliocladium- and verticillium-like *Hypocrea* anamorphs disperse quickly in KOH or water. Gliocladium- and verticillium-like morphologies have convergently evolved or been lost multiple times in the evolution of *Hypocrea/Trichoderma* and it can be hypothesized that they represent an adaptation facilitating dispersal by water or by insects. In the latter case, efficacy would be conferred by the slimy, sticky nature of the conidial masses.

Species complexes in *Hypocrea/Trichoderma*

Species complexes may be common in *Hypocrea/Trichoderma*. In many cases, even though "phylogenetic species" are observed, no discrete character or set of characters was found that could be employed to create a morphologically based taxonomy. This problem affects, for example, the *H. lixii/T. harzianum* species complex (Chaverri *et al.* 2003b). Numerous studies have been published in which the taxonomy of fungal groups has been revised based primarily on results of phylogenetic DNA sequence analysis. In most cases, phenotypic characters to distinguish the "phylogenetic species" were found and the species were described based on these characters. Examples of this process include studies on *T. aggressivum* (Samuels *et al.* 2002), *Cryptococcus neoformans* var. *grubyi* (Franzot *et al.* 1999), *Gibberella fujikoroi* species complex (Nirenberg & O'Donnell 1998, O'Donnell *et al.* 1998), and *Fusarium hostae* (Geiser *et al.* 2001). In the present study, the CHLOROSPORA subgroup was initially considered a species complex. The included species are phylogenetically and morphologically very similar. However, within this group we found that *H. cremea, H. surrotunda, H. sinuosa, H. chlorospora,* and *H. thelephoricola* each form a monophyletic group supported by high bootstrap values in both EF-1α and RPB2 studies (Figs. 2–7). We also found discrete anamorphic and colonial morphology characters and growth rate differences that can be used to distinguish them (see Table 6, Fig. 8). On the other hand, the RPB2 and EF-1α sequences obtained within species such as *H. virens/T. virens* (Chaverri *et al.* 2001a), *H. chromosperma*, *H. strictipilosa/T. strictipile* (Chaverri *et al.* 2003a), and *H. cinnamomea* show less variable or almost identical RPB2 and EF-1α sequences within species (data not shown; but sequences are deposited in GenBank). Samuels and collaborators are in the process of performing parallel studies on the *H. koningii*/*T. koningii* and *H. rufa/T. viride* species complexes, teleomorphs of which have hyaline ascospores.

Genetics of *Hypocrea* species with green ascospores and associated *Trichoderma* species

The production of fertile stromata in artificial conditions in *Hypocrea/Trichoderma* is rare. Among the few species that have been found to produce stromata and ascospores in culture are *H. citrina*, *H. citrina* var. *americana* Canham, *H. psychrophila*, and *H. pulvinata,* which have hyaline ascospores (Canham 1969), and the green-ascospored *H. cuneispora* (Chaverri *et al.* 2003a), *H. nigrovirens* (Chaverri *et al.* 2001b), and *H. spinulosa* (Mathieson 1952). Because these cultures were isolated from single ascospores, it is not known if the formation of fertile stromata is a result of homothallism or mating-type switching; a phenomenon that has been reported for *H. spinulosa*.

Mathieson (1952) and Perkins (1987) stated that sexual reproduction in *H. spinulosa* was under the control of one locus with two alleles, but half of the progeny appeared to be self-sterile. This could be a result of mating-type switching, *i.e.* of a unidirectional shift yielding the compatible mating type in some mycelial nuclei. Chaverri *et al.* (2003b) did preliminary mating compatibility tests in the *H. lixii/T. harzianum* species complex. They found that mycelium of all the isolates in this complex intermingled when crossed or selfed, but only one combination produced stromata with fertile ascospores.

Towards a species concept in *Hypocrea/ Trichoderma*

One of the purposes of systematics is to identify hierarchical taxonomic levels (*e.g.* species) that reflect the evolution of the organisms. Over the course of scientific history, taxonomists and evolutionists have attempted to define species concepts using several approaches. The best-known types of species concepts include the morphological (Hawksworth *et al.* 1995, John & Maggs 1997), Biological (Mayr 1940), phylogenetic (Cracraft 1983), and evolutionary species concepts (Simpson 1951, 1961, Wiley 1978). These will be abbreviated here as MSC, BSC, PSC, and ESC, respectively.

Table 6. Diagnostic phenotypic characters in the CHLOROSPORA subgroup.

Character	***chlorospora***	***cremea***	***sinuosa***	***surrotunda***	***thelephoricola***	***costaricensis***
Substratum	Well-rotten decorticated wood	Decorticated wood	Well-rotten decorticated wood	Decorticated wood	Basidiomycete	Decorticated wood
Part-ascospores						
Shape	monomorphic	dimorphic	monomorphic	dimorphic	dimorphic	dimorphic
Distal (µm)	5.0–5.2 × 4.8–5.2	5.5–6.0 × 5.2–5.5	5.5–5.7 × 5.0–5.5	5.0–5.5 × 5.0–5.5	3.5–3.7 × 3.5–3.7	5.5–5.7 × 5.5–5.7
Proximal (µm)	5.0–5.3 × 4.5–4.7	5.7–6.2 × 4.7–5.2	5.5–5.7 × 4.7–5.0	4.7–5.2 × 4.5–4.7	3.8–4.0 × 3.0–3.2	5.5–6.0 × 5.2–5.7
Formation of pustules on						
CMD	+	+	+	+	-	-
PDA	+	-	+	-	-	-
Conidiophore type	trichoderma-pachybasium-like	trichoderma-like	trichoderma-like	pachybasium-like	gliocladium-like	verticillium-like
Phialides shape	hooked	straight	hooked	straight	straight	
Size (µm)	7.7–8.0 × 4.0–4.2	10.5–12.7 × 2.7–3.2	7.5–8.0 × 3.5–3.7	8.5–9.5 × 3.7–4.0	12.0–13.5 × 2.5–2.7	16.0–25.5 × 2.7–4.0
L/W	1.9–2.0	3.5–4.2	2.0–2.2	2.2–2.6	4.6–5.3	5.5–7.2
Conidia						
Size (µm)	4.0–4.3 × 3.5–3.8	4.0–4.5 × 3.5–3.7	4.5–4.7 × 3.5–3.7	4.5–5.0 × 3.7–4.0	4.0–4.5 × 2.8–3.0	5.2–6.0 × 3.2–4.0
L/W	1.1–1.2	1.1–1.2	1.2–1.3	1.2–1.3	1.4–1.6	1.5–1.8
Formation of chlamydospores	-	-	+	-	-	++
Colony radius (mm) on PDA after 3 d at:						
15 °C	12–20	14–19	4–18	9–14	0–2	5–7
20 °C	32–42	47–50	26–43	18–20	2–9	11–13
25 °C	51–60	63–72	41–58	26–32	6–16	16–17
30 °C	52–62	70–74	45–60	20–22	13–15	15–18
35 °C	1–3	4–5	0–3	0	0–1	7–8
Colony radius (mm) on SNA after 3 d at:						
15 °C	12–16	12–21	3–12	2–7	1	4–6
20 °C	27–37	27–48	21–28	6–14	3–4	9–12
25 °C	40–46	42–55	29–36	12–24	4–10	11–13
30 °C	43–47	55–63	29–39	19–26	4–7	11–15
35 °C	1–2	2–7	0–2	0	0	7–8

The most extensively used concept is the MSC, which has been described as requiring a discrete character or group of characters that can be used to distinguish a species. Generally, this concept is not very effective for most groups of fungi because modern studies have shown that most morphological characters are a result of convergent evolution. For example, description of species of *Hypocrea* based only on teleomorph characters is ineffective because the morphology of the stroma and ascospores is highly conserved, that is, there is little variation in the stroma colour and shape, and ascospore size and shape. For example, in the CHLOROSPORA subgroup, *H. strictipilosa, H. catoptron, H. straminea, H. cuneispora, H. chromosperma* all have yellowish stromata but are not phylogenetically closely related. *Trichoderma* species can be used to illustrate another problem with morphological species concepts, in that they show a high level of phenotypic heterogeneity in which quantifiable characters are lacking, leading to a morphological continuum within which it is difficult to circumscribe species. A dramatic example is seen in the *H. lixii/T. harzianum* species complex.

The BSC recognizes a species as a group of interbreeding populations that are reproductively isolated from other groups. This approach has been useful for taxa that can produce fruiting bodies in the laboratory as a result of mating tests, such as *Neurospora* species (Shear & Dodge 1927) and basidiomycetes such as *Armillaria, Pleurotus,* and *Omphalotus* (Petersen & Hughes 1999). As mentioned above, Chaverri *et al.* (2003b) crossed several isolates of *H. lixii/T. harzianum* to see if phylogenetic lineages correlated with biological groups and found that the crosses did not produce fertile fruiting bodies, with one exception. The only successful mating involved isolates of the same phylogenetic lineage. Unfortunately, mating tests in most Ascomycetes, including some species of *Hypocrea/Trichoderma*, generally have not been successful. Therefore, the BSC is generally not a viable option to distinguish species for most ascomycetes.

The PSC was defined as the "...smallest diagnosable cluster [clade] of individual organisms within which there is a pattern of ancestry and descent. " (Cracraft 1983). Several mycological studies have proposed revised taxonomies based on the PSC, as delimited using molecular sequence data. This has been done, for example, within the *Gibberella fujikoroi* species complex (Nirenberg & O'Donnell 1998, O'Donnell *et al.* 1998) and within the *Aspergillus flavus* complex (Geiser *et al.* 2000). In many cases where such studies were done, even though clades were found, no diagnosable phenotypic characters were found to correlate with these clades, as is seen in *H. lixii/T. harzianum* (Chaverri *et al.* 2003b). An estimated 25 % of the published studies on fungal molecular systematics/phylogenetics have not proposed a formal taxonomy, in part and perhaps in response to this phenotypic species diagnosis problem (Hibbett & Donoghue 1998). Many of these studies were based on a single gene; however, the value of this approach has been cast into doubt (O'Donnell & Cigelnik 1997, O'Donnell *et al.* 1998, Lieckfeldt & Seifert 2000, Taylor *et al.* 2000). Nowadays, many molecular phylogenetic studies are basing their taxonomic proposals on analyses of multiple genes, as suggested by Taylor *et al.* (2000) in his advocacy of "genealogical concordance phylogenetic species recognition" (GCPSR).

The ESC was reviewed by Mayden (1997) and presented as the only species concept that was primarily theoretical. The ESC was described as defining "...a single lineage of ancestor-descendent populations which maintains its identity from such other lineages and which has its own evolutionary tendencies and historical fate" (Wiley 1978). Mayden gave preference to this concept, even though it entailed no particular recognition criteria.

Several recent studies on the systematics of *Hypocrea/Trichoderma* have proposed a taxonomy based on the combination of phenotypic and genotypic characters. As mentioned before, this approach was used in studies on the *H. schweinitzii* Complex (Samuels *et al.* 1998) and on the "green mold" of commercial mushrooms (Samuels *et al.* 2002), as well as in the distinction of certain morphologically similar species (Lieckfeldt *et al.* 1999, Samuels *et al.* 1999, Dodd *et al.* 2002, Chaverri *et al.* 2003a). We are trying to develop a species concept of *Hypocrea/Trichoderma* (based on the results presented in this study) as a combination of the GCPSR and MSC, which can be defined as the smallest diagnosable phylogenetic lineage (clade) that can be distinguished by discrete phenotypic character or characters. This species concept attempts to reflect true evolutionary relationships and make species diagnosis practical to naturalists, biologists, or mycologists who do not have access to molecular phylogenetic methodologies. There are still many more species of *Hypocrea/Trichoderma* to be found and described, especially in unexplored places and niches. This study strives to be a significant contribution in the effort to monograph the "phylogenetic genus" *Hypocrea/Trichoderma*.

DICHOTOMOUS KEYS

Key to *Hypocrea* species with green ascospores based on teleomorphs[1]

1. Stromata formed of loosely interwoven hyphae, readily forming in culture; no conidiophores observed in culture ..... ***H. spinulosa* (28)**
Stromata formed of pseudoparenchymatous tissue, perithecia rarely formed in culture; rarely on herbaceous substrata **2**

2. Part-ascospores on average ≥ 8.5 µm diam **3**
Part-ascospores on average < 8.5 µm diam **4**

3. Stromata pulvinate, with whitish tomentum on the surface, 1.0–2.5 mm diam; distal part-ascospores 11.4–13.0 × 11.0–12.5 µm, proximal part-ascospores 11.5–13.5 × 10.8–13.2 µm ..... ***H. macrospora* (22)**
Stromata tuberculate, hirsute, 0.6–0.9 mm diam; distal part-ascospores 10.2–11.5 × 9.5–10.5 µm, proximal part-ascospores 9.5–10.7 × 8.5–9.0 µm ..... ***H. tuberosa* (37)**

4. Distal part-ascospore on average > 7.0 µm long **5**
Distal part-ascospore on average < 7.0 µm long **8**

5. Stromata light orange, KOH–; ascospores dimorphic, distal part subglobose to ellipsoidal, (6.0–)7.0–8.2(–9.0) × (5.5–)6.5–7.0(–8.5) µm; proximal part wedge-shaped to ellipsoidal, (6.2–)7.0–8.0(–10.0–) × (5.0–)5.7–7.0(–8.5) µm; known only from Czech Republic ..... ***H. velenovskyi* (38)**
Stromata and ascospores not as above; known from Asia or Neotropics **6**

6. Stromata greyish-yellow to yellow-orange, KOH+; ascospores monomorphic, globose to subglobose, distal part-ascospores 7.0–7.5 × 6.5–7.0 µm, proximal part-ascospores 6.7–7.5 × 6.2–6.7 µm; Neotropical ..... ***H. virescentiflava* (40)**
Stromata and ascospores not as above; Asia **7**

7. Stromata pale greyish yellow, 1–2 mm diam, KOH+; distal part-ascospores 7.3–8.0 × 5.8–6.0 µm, proximal part-ascospores 7.1–7.6 × 5.5–6.0 µm ..... ***H. centristerilis* (6)**
Stromata greyish yellow to greyish orange, 2–12 mm diam, KOH–; distal part-ascospores 7.2–8.2 × 6.2–7.0 µm, proximal part-ascospores 7.5–8.5 × 5.7–6.5 ..... ***H. albocornea* (9)**

8. Distal part-ascospores 6.5–7.0 µm long, proximal part-ascospore 7.0–7.7 µm long; stromata brownish-orange, KOH+ ..... ***H. cuneispora/ T. cuneisporum* (17)**
Distal and proximal part-ascospores on average <6.5 µm long **9**

9. Part-ascospores monomorphic, almost globose; stromata in various shades of yellow **10**
Part-ascospores dimorphic, globose, subglobose, wedge-shaped, or subcylindrical; stromata of various colours **14**

10. Part-ascospores on average 3.3–4.0 µm diam **11**
Part-ascospores on average 4.5–5.7 µm diam **12**

11. Stromata yellow to brownish orange, 1.5–2.5 mm diam, somewhat flattened; ascospores slightly warted, part-ascospores 3.5–4.0 µm diam; known only from Northern Europe ..... ***H. aureoviridis/ T. aureoviride* (3)**
Stromata pale greyish yellow, 0.7–1.0 mm diam, pulvinate; ascospores warted, part-ascospores 3.3–3.5 µm diam; known only from Costa Rica ..... ***H. candida/T. candidum* (4)**

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[1] **Values given represent 95% confidence intervals**

12. Stromata yellowish, opaque, KOH+; anamorph gliocladium-like ..... ***H. virens/T. virens*** **(39)**
Stromata yellowish, waxy, somewhat transparent, KOH–; anamorph not gliocladium-like, with sinuous conidiophores and branches **13**

13. Stromata generally gregarious, with a constricted base; distal part-ascospores 5.5–5.7 × 5.0–5.5 µm, proximal part-ascospores 5.5–5.7 × 4.7–5.0 µm; conidiophores and branches narrow, phialide L/W 2.0–2.2, conidia length 4.5–4.7 µm, conidia L/W 1.2–1.3; colony radius on SNA after 3 d at 25 ºC 29–36 mm, at 30 ºC 29–39 mm ***H. sinuosa/T. sinuosum*** **(27)**
Stroma generally not gregarious, not constricted at the base; distal part-ascospores 5.0–5.2 × 4.8–5.2 µm, proximal part-ascospores 5.0–5.3 × 4.5–4.7 µm; conidiophores and branches somewhat wide, phialide L/W 1.9–2.0, conidial length 4.0–4.3 µm, conidial L/W 1.1–1.2; colony radius on SNA after 3 d at 25 ºC 40–46 mm, at 30 ºC 43–47 mm ..... ***H. chlorospora/ T. chlorosporum*** **(9)**

14. Distal part-ascospores on average < 4.0 µm diam **15**
Distal part-ascospores on average > 4.0 µm diam **18**

15. On decaying leaves of *Clusia* sp.; stromata KOH+; distal part-ascospores 2.8–3.0 × 2.5–2.7 µm..... ***H. clusiae*** **(12)**
Not on leaves; stromata KOH–; distal part-ascospores 2.5–3.7 µm diam **16**

16. Stromata dark brown almost black, 6–15 mm diam, surface glabrous, coriaceous, tissue composed of very thick-walled cells; distal part-ascospores 3.5–3.7 × 3.0–3.1 µm ..... ***H. cornea*** **(13)**
Stromata whitish to pale yellow, 0.6–7.8 mm diam, surface smooth or tomentose, tissue not coriaceous **17**

17. Stromata white, 1.8–7.8 mm diam, surface tomentose, distal part-ascospores 2.5–3.3 µm diam; distinct macroconidia present; on wood; known from Indonesia ..... ***H. sulawesensis*** **(32)**
Stromata pale yellow, 0.6–0.8 mm diam, surface hirsute, distal part-ascospores 3.5–3.7 µm diam; anamorph gliocladium-like, not forming macroconidia; on hymenium of *Thelephoraceae;* known from U.S.A. ..... ***H. thelephoricola/ T. thelephoricola*** **(36)**

18. Stromata very dark brown or green, often appearing black **19**
Stromata in pale shades of yellow, brown and orange **21**

19. Stromata 2.5–5.0 mm diam; distal part-ascospores 5.0–5.0 × 4.5–4.8 µm; anamorph gliocladium-like (similar to *G. viride*) ..... ***H. melanomagna/ T. melanomagnum*** **(23)**
Stromata on average < 1.5 mm diam **20**

20. Stromata 1.0–1.5 mm diam, surface smooth; distal part-ascospores 4.3–4.4 × 3.9–4.0 µm; anamorph pachybasium- to trichoderma-like, conidia subglobose ..... ***H. lixii/T. harzianum*** **(21)**
Stromata 0.5–1.0 mm diam, surface tuberculate; distal part-ascospores 6.0–6.5 × 5.5–6.0 µm; anamorph gliocladium-like ..... ***H. nigrovirens/ T. nigrovirens*** **(24)**

21. Stromata yellow to yellow-orange, cylindrical, surface flat to somewhat concave, seated on a thick subiculum ..... ***H. substipitata*** **(31)**
Stromata yellow to brown, not on a thick subiculum and not cylindrical **22**

22. Stromata in shades of brown (but not appearing black) **23**
Stromata in shades of yellow **30**

23. Stromata reddish brown, deeply furrowed or wrinkled; distal part-ascospores 5.2–5.5 × 4.7–5.0 µm, proximal part-ascospores 5.0–6.0 × 4.5–4.7 µm; known only from India and Sri Lanka ..... ***H. rugulosa*** **(26)**
Stromata not deeply furrowed or wrinkled; not known from Sri Lanka or India **24**

24. Distal part-ascospores on average 5.3–6.5 long **25**
Distal part-ascospores on average < 5.3 µm long **26**

25. Stromata KOH+, brownish orange; distal part-ascospores 5.8–6.5 × 4.0–5.0 µm; anamorph pachybasium-like ..... ***H. atrogelatinosa*** **(2)**
Stromata KOH–, brown, violet-brown, or dark reddish brown; distal part-ascospores 5.3–5.5 × 4.5–5.0 µm; anamorph verticillium-like ..... ***H. tawa/T. tawa*** **(34)**

26. Stromata brownish orange, 0.5–0.7 mm diam, waxy, somewhat transparent; distal part-ascospores 4.3–4.5 x 4.0–4.2 µm, proximal part-ascospores 4.8–5.2 × 3.5–3.7 µm; anamorph gliocladium-like, phialides 10.5–11.5 × 3.3–3.5 µm (L/W 3.1–3.5), conidia conspicuously held in drops of clear green liquid ..... ***H. gelatinosa/T. gelatinosum*** **(19)**
Stromata characters not in above combination; conidia apparently held in dry heads **27**

27. Stromata reddish brown (brick red), opaque, 0.8–1.7 mm diam; distal part-ascospores 3.9–4.7 × 3.4–4.2 µm, proximal part-ascospores 4.2–5.2 × 3.1–3.7 µm; anamorph pachybasium-like, conidia 3.3–3.7 × 2.7–3.0 (L/W 1.1–1.3) ..... ***H. ceramica/T. ceramicum*** **(8)**
Stromata and anamorph characters not in above combination **28**

28. Stromata brown to light brown, KOH–, opaque; phialides 9.0–10.3 × 3.7–4.0 µm, conidia 4.2–4.5 × 3.3–3.5 µm ..... ***H. cinnamomea/T. cinnamomeum*** **(11)**
Stromata brownish orange or pale to dark reddish brown, KOH+, waxy, semitransparent; phialides 6.5–8.0 µm long,conidia 3.0–3.7 × 2.3–3.2 µm **29**

29. Distal part-ascospores 4.5–4.7 × 4.0–4.2 µm, proximal part-ascospores 4.8–5.0 × 3.5–3.7 µm; anamorph pachybasium- to gliocladium-like, conidia 3.4–3.7 × 3.0–3.2 µm; known only from U.S.A. ..... ***H. ceracea/T. ceraceum*** **(7)**
Distal part-ascospores 3.5–4.0 × 3.5–4.0 µm, proximal part-ascospores 4.0 × 3.0–3.5 µm; anamorph pachybasium-like, conidia 3.0 × 2.3–2.5 µm; known only from France ..... ***H. phyllostachydis/T. phyllostachydis*** **(25)**

30. Stromata KOH+ **31**
Stromata KOH– **36**

31. Distal part-ascospores on average < 4.5 µm long, proximal part-ascospores 4.2–4.7 × 3.5–3.8 µm **32**
Distal part-ascospores on average ≥ 4.5 µm long, proximal part-ascospores on average > 4.5 µm diam **33**

32. Stromata generally gregarious, pale yellow, 0.9–1.2 mm diam; distal part-ascospores 4.2–4.3 × 4.0–4.2 µm, proximal part-ascospores 4.2–4.5 × 3.6–3.8 µm; anamorph with few phialides formed; phialides hooked or twisted; conidia pale green; known only from U.S.A. ..... ***H. chromosperma/T. chromospermum*** **(10)**
Stromata not gregarious, yellowish brown, 1.4–2.1 mm diam; distal part-ascospores 4.0–4.3 × 3.8–4.0 µm, proximal part-ascospores 4.5–4.7 × 3.5–3.7 µm; anamorph with pachybasium- and gliocladium-like branches, abundant phialides; phialides straight, conidia green; known only from Thailand ..... ***H. thailandica/T. thailandicum*** **(35)**

33. Distal part-ascospores 4.5–5.0 × 3.7–4.0 µm, proximal part-ascospores 4.5–5.3 × 3.2–3.7 µm **34**
Distal part-ascospores 5.0–5.5 µm diam, proximal part-ascospores 5.0–6.2 × 4.2–5.2 µm **35**

34. Stromata 1–4 mm diam, greyish yellow to greyish orange; known only from Brazil ..... ***H. gyrosa*** **(20)**
Stromata 0.1–1.0 mm diam, pale yellow to greyish yellow; known only from Sri Lanka ..... ***H. straminea/T. stramineum*** **(29)**

35. Phialides 14.7–18.2 × 2.5–3.0 µm (L/W 5.4–7.9); conidia 4.0–4.2 × 3.2–3.5 µm ***H. estonica/T. estonicum*** **(18)**
Phialides 7.2–7.7 × 4.0–4.2 µm (L/W 1.8–2.0); conidia 4.5–4.7 × 3.5–3.7 µm ***H. strictipilosa/T. strictipile*** **(30)**

36. Anamorph verticillium-like; phialides 16.0–25.5 × 2.7–4.0 µm (L/W 5.5–7.2) ..... ***H. costaricensis*** **(14)**
Anamorph trichoderma- or pachybasium-like; phialides 5.5–13.0 × 2.7–4.2 µm (L/W 1.4–4.2) **37**

37. Phialides 5.5–7.2 × 3.2–4.2 µm (L/W 1.4–2.2); conidia 3.5–4.0 × 2.3–2.7 µm (L/W 1.4–1.6) ..... ***H. catoptron/T. catoptron*** **(5)**
Phialides 8.5–13.0 × 2.7–4.0 µm (L/W 2.2–4.2); conidia 4.0–5.0 × 3.5–4.0 µm (L/W 1.1–1.3) **38**

38. Stromata 0.9–1.2 mm diam; distal part-ascospores 5.5–6.0 × 5.2–5.5 µm, proximal part-ascospores 5.7–6.2 × 4.7–5.2 µm; phialides 10.5–12.7 × 2.7–3.2 µm (L/W 3.5–4.2); conidia 4.0–4.5 × 3.5–3.7 µm (L/W 1.1–1.2) ..... ***H. cremea/T. cremeum*** **(6)**
Stromata 1.5–2.6 µm; distal part-ascospores 5.0–5.5 × 5.0–5.5 µm, proximal part-ascospores 4.7–5.2 × 4.5–4.7 µm; phialides 8.5–9.5 × 3.7–4.0 µm (L/W 2.2–2.6); conidia 4.5–5.0 × 3.7–4.0 µm (L/W 1.2–1.3) ..... ***H. surrotunda/ T. surrotundum*** **(3)**

Key to *Hypocrea* species with green ascospores based on anamorph characters

1. Conidia on average > 5 µm long, green **2**
Conidia on average < 5 µm long, green **6**

2. Anamorphs gliocladium-like; conidia 6.0–6.5 × 4.5–5.0 µm **3**
Anamorphs verticillium- or pachybasium-like; conidia 5.0–6.0 × 3.2–4.0 µm **4**

3. Conidiophores generally arising from the surface of the agar; phialides 13.5–15.5 × 3.5–4.0 µm (L/W 3.5–4.0); stromata black, strongly tuberculate ..... ***H. nigrovirens/T. nigrovirens*** **(24)**
Conidiophores arising from aerial hyphae; phialides 13.5–15.7 × 4.3–4.6 (L/W 3.1–3.6); conidia 6.0–6.5 × 4.7–5.0 µm (L/W 1.3); pustulate anamorph sometimes formed; phialides from pustulate anamorph 4.4–9.5 × 3.0–4.2 µm; conidia from pustulate anamorph 3.7–5.3 × 2.6–3.7 µm; stromata yellowish, pulvinate, not tuberculate ..... ***H. crassa/T. crassum*** **(15)**

4. Anamorph pachybasium-like, conidiophore elongations present, fertile or not, verticillium-like synanamorph rarely present; phialides 6.0–7.2 × 3.7–4.0 µm ..... ***H. cuneispora/ T. cuneisporum*** **(17)**
Anamorphs verticillium-like, conidiophore elongations absent; phialides 14–26 × 2.7–4.0 µm..... **5**

5. Phialides 16.0–25.5 × 2.7–4.0 µm (L/W 5.5–7.2); conidia 5.2–6.0 × 3.2–4.0 µm (L/W 1.5–1.8); abundant chlamydospores formed; stromata pale yellow ..... ***H. costaricensis*** **(14)**
Phialides 14.0–16.0 × 3.0–3.2 µm (L/W 4.7–5.5); conidia 5.2–6.0 × 3.5–3.7 µm (L/W 1.4–1.6); no chlamydospores formed; stromata brown, brown-violet or orange-brown ..... ***H. tawa/T. tawa*** **(34)**

6. Anamorph forming micro- and macro-conidia; micro-conidia green, aseptate, ellipsoidal; macro-conidia hyaline, subulate, with a rounded, somewhat thick-walled basal cell, often multiseptate ..... ***H. sulawesensis*** **(32)**
Anamorphs not forming macro-conidia **7**

7. Anamorphs gliocladium-like **8**
Anamorphs trichoderma-, pachybasium-, or verticillium-like **11**

8. Conidiophores formed in irregular fascicles throughout the plate; phialides 10.5–11.5 × 3.3–3.5 µm (L/W 3.1–3.5); conidia 4.0–4.3 × 3.3–3.5 µm (L/W 1.2–1.3), held in drops of clear green liquid ..... ***H. gelatinosa/ T. gelatinosum*** **(19)**
Conidiophores not in fascicles, rather arising singly from the agar or from aerial hyphae; phialides on average >11.5 µm long **9**

9. Conidiophores generally arising from aerial hyphae; phialides 8.8–9.2 × 4.0–4.2 µm (L/W 2.2–2.3); conidia 4.5–4.7 × 3.8–4.0 µm (L/W 1.2), held in drops of clear green liquid; abundant chlamydospores formed ..... ***H. virens/T. virens*** **(39)**
Phialide L/W > 4.5; conidia 3.2–3.7 × 2.5–3.2 µm **10**

10. Discrete conidiophore, branching 3–4 times (penicillus ter- to quaterverticillate); phialides 11.5–13.0 × 1.7–2.0 µm (L/W 5.8–6.8); conidia 3.3–3.7 × 2.5–2.7 µm (L/W 1.3–1.4); anamorph similar to *Gliocladium viride*; stromata black, 2.5–5.0 mm diam; on decorticated wood ..... ***H. melanomagna/T. melanomagnum*** **(23)**

More or less discrete conidiophores, branching 2–3 times (penicillus bi- to terverticillate); phialides 12.0–13.5 × 2.5–2.7 µm (L/W 4.6–5.3); conidia 4.0–4.5 × 2.8–3.0 µm (L/W 1.4–1.6); stromata yellowish, 0.6–0.8 mm diam; on hymenium of *Thelephoraceae* ..... ***H. thelephoricola/T. thelephoricola*** **(36)**

11. Anamorph odd gliocladium-like; conidiophores with verrucose base, generally with very few phialides formed; phialides lageniform, more often hooked or twisted, 6.9–14.5 × 2.5–3.5 µm (L/W 2.5–4.8); conidia pale green, 4.0–6.5 × 3.7–3.8 µm (L/W 1.4–1.7); found in Eastern U.S.A. ..... ***H. chromosperma/T. chromospermum*** **(10)**
Anamorph characteristics not in the above combination **12**

12. Pachybasium-like anamorphs with conidial L/W > 1.4 on average **13**
Various types of anamorphs with conidial L/W < 1.4 on average **15**

13. Conidia 3.0–3.2 × 2.0–2.2 µm (L/W 1.4–1.5); sinuous conidiophore elongations present; verticillium-like synanamorph present; phialides 4.7–5.0 × 3.0–3.2 µm; known only from Sri Lanka ..... ***H. straminea/T. stramineum*** **(29)**
Conidia on average > 3.5 µm long and >2.5 µm wide **14**

14. Gliocladium-like synanamorph formed abundantly in aerial hyphae; phialides from pustulate anamorph 4.4–9.5 × 3.0–4.2 µm; conidia 3.7–5.3 × 2.6–3.7 µm (L/W 1.4); phialides from gliocladium-like anamorph 13.5–15.7 × 4.3–4.6 µm; conidia 5.9–6.4 × 4.7–4.9 µm ..... ***H. crassa/T. crassum*** **(15)**
Verticillium-like synanamorph formed in areas of effuse conidiation; phialides of pachybasium-like anamorph 5.5–7.2 × 3.2–4.2 µm; conidia 3.5–4.0 × 2.3–2.7 µm (L/W 1.4–1.6) ..... ***H. catoptron/T. catoptron*** **(5)**

15. Phialides on average > 8.5 µm long **16**
Phialides on average < 8.5 µm long **21**

16. Phialides on average <15 µm long **17**
Phialides 14.7–18.2 × 2.5–3.0 µm (L/W 5.4–7.9); conidia 4.0–4.2 × 3.2–3.5 µm (L/W 1.2–1.3); stromata pale yellow to greyish yellow, KOH+ ..... ***H. estonica/T. estonicum*** **(18)**

17. Anamorph pachybasium-like; phialide L/W < 2.9 on average **18**
Anamorph trichoderma-like; phialide L/W > 2.9 on average **19**

18. Phialides 9.0–10.3 × 3.7–4.0 µm (L/W 2.4–2.8), conidia 4.2–4.5 × 3.3–3.5 µm (L/W 1.2–1.3); colony radius on PDA at 25 ºC after 3 d 2–6 mm; stromata brown to light brown ..... ***H. cinnamomea/T. cinnamomeum*** **(11)**

Phialides 8.5–9.5 × 3.7–4.0 µm (L/W 2.2–2.6); conidia 4.5–5.0 × 3.7–4.0 µm (L/W 1.2–1.3); colony radius on PDA at 25 ºC after 3 d 26–32 mm; stromata pale yellow ..... ***H. surrotunda/T. surrotundum*** **(33)**

19. Phialides 10.5–12.7 × 2.7–3.2 µm; conidia 4.0–4.5 × 3.5–3.7 µm (L/W 1.1–1.2); colony radius on PDA at 25 ºC after 3 d 63–72 mm; stromata pale yellow ..... ***H. cremea/T. cremeum*** **(16)**
Conidia on average <4.0 µm long and <3.5 µm wide; colony radius on PDA at 25 ºC after 3 d on average < 35 mm; stromata in shades of yellow or brownish orange **20**