

# THE DEVELOPMENT OF HOLOMORPHIC CONCEPTS IN OPHIOSTOMATALEAN ASCOMYCETES

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## SUMMARY

The ophiostomatoid fungi including the genera *Ceratocystis* s.str., *Ophiostoma* and *Ceratocystiopsis* have strong anamorph/teleomorph connections and provide an outstanding model for evaluating taxonomic schemes based on morphology. Recent evidence from extended collections as well as ultrastructural and molecular studies suggests that these fungi have evolved several, if not many times. Primary taxonomic characters for the group such as ascomatal structure and ascospore shape now appear to have been misleading. A great deal of convergence has evidently occurred and re-evaluation of morphological characters based on additional molecular studies is needed before a new and more meaningful classification can be established for this group. Ultimately, we expect that numerous genera, belonging to at least two and perhaps more distantly related groups of fungi will emerge.

## INTRODUCTION

The ophiostomatoid fungi including the well known genera *Ceratocystis* and *Ophiostoma* have been known since early this Century. They include many important plant pathogens particularly of trees, symbionts of insects, species of medical importance as well as species with potential industrial and biotechnological significance (Wingfield et al., 1993). The taxonomy of the ophiostomatoid fungi has been the subject of considerable controversy, virtually since their initial discovery. This controversy continues today and there are many conflicting opinions concerning the subdivision of the group, not only at the generic and species levels but also at higher taxonomic rankings (Upadhyay, 1993).

*Ceratocystis* s.lat. encompasses approximately 116 species of superficially similar ascomycetes which may be only distantly related. This taxonomic aggregate is commonly subdivided into three genera: *Ceratocystis* s.str., *Ophiostoma* and *Ceratocystiopsis* (de Hoog and Scheffer, 1984; Wingfield et al., 1993). *Ceratocystis* s.str. can be distinguished from the other two genera by four features: (1) the presence of *Chalara* anamorphs with ring wall building conidial development (Minter et al., 1982); (2) the absence of rhamnose and cellulose in cell walls (Rosinski and Campana, 1964; Jewell, 1974; Weisman and de Hoog, 1975);

(3) sensitivity to even low concentrations of the antibiotic cycloheximide (Harrington, 1981); (4) an association with non-specific insect vectors such as flies and nitidulid beetles (Dowding, 1973; Juzwik and French, 1983). In contrast, *Ophiostoma* and *Ceratocystiopsis* species have: (1) anamorphs in a number of genera such as *Leptographium*, *Graphium*, *Sporothrix* and *Hyalorhinocladiella* with apical wall building conidial development; (2) rhamnose and cellulose in their cell walls; (3) tolerance to high levels of cycloheximide; (4) in many cases, an association with specific insect vectors such as scolytid (*Coleoptera: Scolytidae*) bark beetles (Upadhyay, 1981). *Ceratocystiopsis* has been separated from *Ophiostoma* on the basis of its falcate ascospores although this subdivision is beset with a number of incongruities (Wingfield, 1993).

The major characteristic unifying the ophiostomatoid fungi is an ascoma usually with a neck of variable length containing evanescent asci and ascospores that are extruded from the ascomata in gloeoid masses. They are thus elegantly adapted to insect dispersal and often co-exist with their specific arthropod vectors. Adaptation to insect dispersal has apparently resulted in convergence and the assumption of superficial morphological similarity of ascomata in even distantly related fungi. This problem is further complicated by the presence of anamorph genera typical of ophiostomatoid fungi but also found with connections to other unrelated teleomorphs or of unknown affinity. Dependence on superficial morphological characteristics in taxonomic schemes has therefore led to a great deal of confusion regarding the phylogeny of these fungi.

The ophiostomatoid fungi include outstanding examples of contemporary problems in the taxonomy of the ascomycetes. The aim of this chapter is to illustrate some of these examples, provide a summary of recent developments and to sketch a perspective of future prospects.

## ANAMORPHS OF *CERATOCYSTIS* S.LAT.

Anamorph states have had a considerable influence on the taxonomy of ophiostomatoid fungi. Munch (1907) recognized the presence of "endoconidia" in some species of *Ceratocystis* s.lat. and established *Endoconidiophora* for *E. coerulescens* because it had a *Chalara* anamorph (i.e. *Chalara ungeri*). Similarly, Melin and Nannfeldt (1934) recognized two groups within *Ophiostoma* based on the presence of *Chalara* anamorphs in one and anamorphs that produce conidia exogenously in the other.

Anamorph species currently accommodated in *Ophiostoma* and *Ceratocystiopsis* have had a tremendously complex taxonomic history. Goidanich (1936) recognized that species of *Ophiostoma* with *Leptographium* anamorphs in the *Graphium/Sporothrix* complex were distinct from those with *Chalara* anamorphs and established *Grosmannia* for the former. The introduction of mode of conidium development as a primary taxonomic characteristic by Hughes (1953) led to a proliferation of generic names associated with ophiostomatoid fungi. Hughes (1953) initiated this trend by distinguishing the genus *Verticicladiella* Hughes from *Leptographium* based on sympodial versus percurrent proliferation of conidiogenous cells. Kendrick (1961) followed this lead and established *Phialocephala* for species in the *Leptographium* complex with phialidic conidium development. This trend continued during the 1960's and 1970's and ultimately no fewer than

colorless

18 generic names were recognized for anamorphs of ophiostomatoid fungi (Wingfield et al., 1993; Mouton and Wingfield, 1993). Many of these names were based on analogous anamorphs: colorless versus pigmented; mononematous versus synnematosus; phialidic versus sympodial versus percurrent conidiogenesis.

Wingfield (1985) undertook a comprehensive study of conidium development in species of *Leptographium* and *Verticicladiella* using scanning (SEM) and transmission (TEM) electron microscopy. Results of this study showed that percurrent proliferation of conidiogenous cells was the norm in these fungi and that delayed secession of conidia leads to an illusion of sympodial development when they are viewed using light microscopy (Wingfield, 1985; van Wyk et al., 1988; Mouton et al., 1993a). Subsequent studies on other anamorphs of ophiostomatoid fungi (Wingfield et al., 1987; Wingfield et al., 1991; Mouton et al., 1992; Mouton and Wingfield, 1993; Seifert and Okada, 1993) have also shown that conidium development as interpreted by light microscopy has led to an unnecessary proliferation of names for anamorphs of ophiostomatoid fungi. Electron microscopic studies of conidium development in remaining anamorphs of ophiostomatoid fungi are continuing in our laboratory. We ultimately expect to reduce current names for anamorphs of *Ceratocystis* s.lat. to approximately five reasonably well defined genera including *Chalara*, *Knoxdaviesia*, *Leptographium*, *Graphium*, *Sporothrix* and *Hyalorhinochlaeniella* (Mouton et al., 1993b).

While anamorph characters have been particularly useful in the taxonomy of ophiostomatoid fungi, they have, in some cases also resulted in confusion. For example, ophiostomatoid fungi with *Chalara* anamorphs have easily been segregated in *Ceratocystis* s.str. One exception has been *Ceratocystiopsis falcata* which has a *Chalara* state but, in terms of its teleomorph, is very different from other species of *Ceratocystis* s.str. A similarly enigmatic situation is encountered in *Ceratocystis autographa*. This fungus has a well developed *Chalara* state suggesting that it would best be accommodated in *Ceratocystis* s.str. It, however, is also reported to have a *Sporothrix* state characteristic of typical *Ophiostoma* species. In a recent SEM and TEM study of the anamorphs of *C. autographa* (Benade, 1993), it has been found that the purported *Chalara* state is only superficially similar to that genus. Although it would have been difficult to make these assessments using light microscopy, electron microscopic observations have clearly shown that conidia possess single attachment points and are produced in false chains atypical of *Chalara* (species). The so-called *Chalara* anamorph of *C. autographa* clearly deserves a new generic disposition and we suspect that the same might be true of other anamorphs of *Ceratocystis* s.str.

Convergent evolution and adaptation to transmission by insects has apparently led to the adoption of superficially similar morphologies in distantly related anamorph states. For example, we believe that *Ceratocystis autographa* is more typical of *Ophiostoma* than *Ceratocystis* s.str. Its *Chalara*-like anamorph is in all probability no more than a manifestation of convergence.

A second fascinating example of convergence in ophiostomatoid fungi is encountered in the *Knoxdaviesia* anamorph of *Ceratocystiopsis proteae* which occurs in *Protea* infructescences found only at the southern tip of Africa (Wingfield et al., 1988). In terms of anamorphs of ophiostomatoid fungi, *Knoxdaviesia* superficially resembles *Leptographium* with dark mononematous conidiophores terminating in

gloeoid masses of spores. However, this fungus produces conidia from typical phialidic conidiogenous cells which are unique amongst ophiostomatoid anamorphs (Mouton and Wingfield, 1993). We suspect that *Ceratocystiopsis proteae* and the more recently described *Ophiostoma capense* (Wingfield and van Wyk, 1993) with *Knoxdaviesia* anamorphs from *Protea* infructescences are unrelated to other ophiostomatoid fungi, and their resemblance to these fungi has evolved as an adaptation to insect transmission. Similar examples of convergence might well be found amongst other species of *Ophiostoma* and *Ceratocystiopsis* when less subjective characters than morphology based solely on light microscopy are used to examine these fungi.

Anamorph genera associated with ophiostomatoid fungi are also commonly encountered in distantly related groups of fungi. The genera *Chalara* and *Sporothrix* perhaps provide the best examples of this complex situation. *Chalara* species are, for example found in at least seven ascomycete genera representing five different orders (Nag Raj and Kendrick, 1993) and *Sporothrix* species occur in the *Endomycetes*, *Ascomycetes* and *Basidiomycetes* (de Hoog, 1993). Similarly, many species of these genera that have not been connected to teleomorphs, or simply existing as apparent anamorphic holomorphs, might or might not be related to ophiostomatoid fungi. It is possible that a more detailed examination of these fungi might lead to the discovery of new morphological characters which will enable us to separate them into phylogenetically meaningful groups. Certainly there is sufficient evidence to believe that similar morphological forms have evolved many times and that any attempt to establish natural groups based on these morphs is likely to be beset by many problems.

## TELEOMORPH CHARACTERISTICS

Ascomata of ophiostomatoid fungi are found in niches frequented by insects and many species are formed in the galleries of Scolytid bark beetles with which they are associated (Upadhyay, 1981; Wingfield et al., 1993). They are typically beaked and have necks that vary in length from being virtually non-existent to extremely long. The apex of these necks are often surrounded by ostiolar hyphae that may be straight, divergent or convergent. Ascospores are produced in gloeoid masses and accumulate at the apices of the ascomatal necks facilitating attachment to their vectors.

### Ascomatal morphology

The morphology of the ascomata is the most important characteristic on which the taxonomy of the ophiostomatoid fungi is based. This characteristic has also led to considerable confusion in the classification of the group. Indeed, the first ophiostomatoid fungus to be described was misidentified as a coelomycete, and it was not until the study of Elliott (1925) that this error was corrected. The incorrect placement of this group of fungi resulted from the fact that asci are evanescent and ascospores are extruded from the ascomata without any evidence of the presence of asci.

"Perithecium" is perhaps not a fully appropriate term for the ascomata of ophiostomatoid fungi. Strictly speaking, perithecia are found in pyrenomycetous fungi where asci are formed in unitunicate asci which, in

many cases, have apical structures to facilitate forceful discharge of spores. Indeed, considerable controversy has surrounded the placement of these fungi which have been considered to be either pyrenomycetes because of their long necked ascomata or plectomycetes because of their evanescent and irregularly shaped asci.

The ascomatal form in the ophiostomatoid fungi has obviously evolved in association with insects. Therefore, the ecological pressure has been on these fungi to assume a morphology that will ensure their dispersal. From the taxonomic standpoint this has led to considerable confusion as all fungi with typical ophiostomatoid ascomata have tended to be treated as being phylogenetically related. A common trend has thus been to treat all species in the single genus *Ceratocystis*. Other authors, have accepted one or a number of segregate genera such as *Ophiostoma*, *Ceratocystis* s.str., *Ceratocystiopsis*, and *Europhium* (Wingfield et al., 1993). These have, however, usually been considered as being related and treated in the Family *Ophiostomataceae*, order *Microascales* of the *Plectomycetes* (Upadhyay, 1981).

Contemporary studies have provided substantial evidence to suggest that members of the genus *Ceratocystis* s.str. are phylogenetically distinct from species of *Ophiostoma* and *Ceratocystiopsis* as discussed above. More recently, application of non-subjective molecular techniques have confirmed that species of *Ophiostoma* and *Ceratocystiopsis* are phylogenetically distinct from species of *Ceratocystis* s.str. For example, Hausner et al. (1992), Spatafora and Blackwell (1993; this volume) have compared sequence of the small subunit ribosomal RNA and have shown that *Ceratocystis* is phylogenetically distant from *Ophiostoma*. There also appears to be reasonable congruence between the results of these studies and the results of recent observations on the ultrastructure of centrum development in *Ceratocystis* s.str. and *Ophiostoma* (van Wyk and Wingfield, 1991a, 1991b, 1991c; van Wyk et al., 1991; van Wyk and Wingfield, 1993; van Wyk et al., 1993).

The fascinating group of ophiostomatoid fungi recently discovered in the infructescences of *Protea* species in the Cape Fynbos Biome of South Africa also provide an interesting example of convergent evolution in the ophiostomatoid fungi. The first of these fungi to be described was disposed in the genus *Ceratocystiopsis* as *C. proteae* because it has falcate ascospores (Wingfield et al., 1988). The second species of ophiostomatoid fungus from *Protea* infructescences is very similar to *C. proteae* and also has a *Knoxdaviesia* anamorph (Wingfield and van Wyk, 1993). This fungus is unusual in having ascospores that are not falcate and it has thus been placed in *Ophiostoma* as *O. capense*.

Morphologically, *C. proteae* and *O. capense* are very similar to each other and distinct from *Ceratocystis* s.str. They are, however, sensitive to cycloheximide in growth media which is strong evidence to suggest that they do not reside in *Ceratocystis* s.str. Evidence from molecular studies (Hausner et al., 1993a, 1993b; authors unpubl.) also support the view that these fungi should reside in a genus other than *Ophiostoma*, *Ceratocystis* or *Ceratocystiopsis*. The taxonomic ramifications of these studies will be published later. Of interest in this particular discussion is the fact that the ophiostomatoid fungi with *Knoxdaviesia* anamorphs provide another example of convergent evolution in this group of fungi. All indications are, therefore, that ecological pressure to ensure dispersal by insects has led to similar morphological manifestations in numerous unrelated fungi.

## Ascospore Morphology

Ascospore morphology has been one of the most important characteristics upon which taxonomy of ophiostomatoid fungi has been based. Diverse ascospore forms are encountered in this group, among which galeate or hat-shaped ascospores are perhaps best known. Other than galeate ascospores, species in this group of fungi can have ascospores without sheaths (extended outer wall layers) or with a wide range (inequilateral, orange section, pillow, falcate) of sheath forms (Upadhyay, 1981; van Wyk et al., 1993). These ascospore forms have thus been used as a basis for subdividing *Ceratocystis* s.lat. at the sectional level (Griffin, 1968; Olchowecki and Reid, 1974; Upadhyay, 1981) and even at the generic level in the case of *Ceratocystiopsis* (Upadhyay and Kendrick, 1975; Upadhyay, 1981).

Galeate ascospores are encountered both in *Ceratocystis* s.lat. and in a number of yeast genera. This has been the source of considerable debate as to whether relatedness between these groups of fungi might be implied. For example, Redhead and Malloch (1977) included *Ophiostoma* and *Ceratocystis* with yeasts in the *Endomycetales* primarily on the basis of this character. This grouping was contested by Benny and Kimbrough (1980) because it was based solely on morphology as observed using light microscopy, and did not take into consideration available developmental, ultrastructural and chemical evidence. Von Arx and van der Walt (1987) recognized that the ophiostomatoid fungi were a polyphyletic group yet still contended that the galeate ascospores were indicative of some relatedness. Kendrick et al. (1993) added further commentary on these possible relationships.

Galeate ascospores in *Ceratocystis* and *Ophiostoma* as determined by light microscopy have been the subject of recent ultrastructural study (van Wyk and Wingfield 1991a, 1991b, 1991c; van Wyk et al., 1991; van Wyk et al., 1993). It was determined that the apparently similar galeate ascospores in species of *Ceratocystis* and *Ophiostoma* are fundamentally different in morphology. Ascospores of *Ceratocystis fimbriata* and *C. moniliformis* are typically bowler hat-shaped and develop in pairs (van Wyk et al., 1991). In contrast, those of *Ophiostoma cucullatum* and *O. davidsonii* develop individually, and their brims are triangular (van Wyk and Wingfield, 1991a, 1991b). These observations provide additional evidence that species of *Ceratocystis* and *Ophiostoma* are only superficially similar.

Hausner et al. (1992) have recently considered the evolution of the apparent galeate ascospore using analysis of small subunit ribosomal genes sequences. This study reaffirms the fact that species of *Ceratocystis* and *Ophiostoma* with apparently similar ascospores are distantly related. Furthermore, these authors provide evidence that the ophiostomatoid fungi are also distantly related to yeasts with galeate ascospores.

In an analysis of sequence data derived from ribosomal RNA, Wingfield, Grant, Wolfaardt and Wingfield (1993) have considered the relatedness of various species of *Ceratocystis* s.str. Results of this study have shown that ascospore shape, even within this more clearly defined subgroup of *Ceratocystis* s.lat. is a poor taxonomic character. Indeed, all indications are that *Ceratocystis* s.str. itself represents a phylogenetically diverse group of fungi.

The occurrence of two different ascospore forms in *C. proteae* and *O. capense* which are very similar fungi has led us to examine the ascospores of *C. proteae* more carefully. In an ultrastructural examination of ascospores in this fungus (van Wyk and Wingfield, 1993), it has been shown that the ascospores are reniform without sheaths. The falcate outline detected by light microscopy is apparently an illusion resulting from the adherence of ascomatal remains to the apices of the ascospores when they are released. This study has provided additional evidence that *O. capense* and *C. proteae* are very closely related and should reside in the same genus.

## CONCLUSIONS

The ophiostomatoid fungi are one of our best known groups of fungi with strong anamorph teleomorph connections. Their apparently diagnostic morphology has clearly evolved several if not many times, and thus the taxonomic schemes which we have followed for this group have been wholly misleading. The most important and supposedly conservative taxonomic characters for the ophiostomatoid fungi, ie ascomatal structure and ascospore shape, appear to have been relatively useless. We are now faced with the challenge of finding meaningful groupings and characteristics on which to base a future classification for these fungi.

Although molecular studies have given us very clear indications that the taxonomic schemes that we have followed for the ophiostomatoid fungi in the past have been misleading, we require substantially more data before an ideal classification can be developed. We now know that there has been a great deal of convergence in the evolution of the ophiostomatoid fungi although the exact extent of this must still be determined. Many more examples of these fungi now need to be examined in order to determine the limits of the various natural groups. Once this has been accomplished, we will be in a position to re-evaluate morphological characters and thus to provide a meaningful classification of members of the various groups. At this level, we suspect that numerous genera, belonging to at least two, and perhaps more, distantly related groups of fungi will ultimately result.

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