Reproductive Isolation: Evidence that *Ascobolus stercorarius* and *Ascobolus furfuraceus* are two species, not one.

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Strains obtained from the germination of 30-40 year old ascospores from seven stocks of the heterothallic *Ascobolus stercorarius* - *furfuraceous* complex were mated in all combinations. All *st* x *st* and *fu* x *fu* pairings were fertile (ascospores) whereas all *st* x *fu* pairings were sterile (no ascospores). Based on this reproductive isolation between *st* and *fu* strains I conclude that the complex consists of two species. The block that segregated the two species is probably some stage in the establishment of the dikaryotic phase in ascogenous hyphae. One hypothesis is that this block is at the stage of nuclear recognition.

The taxonomic status of *Ascobolus stercorarius* and *Ascobolus furfuraceus*, two heterothallic members of the family Ascobolaceae, has been in dispute for these many years. Seaver (1942) and Brummelen (1967) treat them as two forms of one variable species. They based their classification on apothecial characters of herbarium specimens. Olive (1954), using living material, concluded that they are two species. He based his conclusion on both morphological differences and reproductive isolation. But vis-à-vis the latter he presented no data, no details, and no observations beyond his conclusion that the two forms are reproductively isolated.

To obtain the missing evidence I went back to two sources of material derived from the Olive collection. The first were two living cultures of stock *st*1, one of *a* mating type, the other of *A*. The second were ascospores of five other *st* stocks (III, IV, V, VI, and VIII) and of one I stock (II). These had been stored dry in test tubes at 4 - 8˚C, some for up to 30 years. The ascospores from each stock were treated by the NaOH 37˚C regimen of Yu (1954) as modified by Bistis (1994). In all cases some of the ascospores germinated. Ten of these from each stock were isolated and cultured. The mating types and mating behavior of these sixty cultures were determined by pairing them with the two vegetative cultures from stock *st* I. (See Bistis, 1956, for details of the crossing method.)

***st* x *st* I**

All fifty of the *st* strains from stocks III, IV, V, VI and VIII produced normal apothecia with one or the other of the two *st* I testers.

***fu* II x *st*I**

None of the ten *fu* II strains produced mature, fertile apothecia when paired with each of the two *st*I testers. Despite this, their mating types could be determined since each of them did interact with one of the testers to produce small, sterile apothecia.

Intersterility

The intersterility between strains of the *st* I and *fu* II stocks as reported by Olive (1954) was confirmed. To study the phenomenon further, 14 strains, one of each mating type from each of the seven stocks, were paired in all 105 combinations.

The integrity of the mating type functions was upheld. No interaction was seen in any of the 56 *A* x *A* and *a* x *a* pairings. On the other hand, all 49 of the *A* x *a* combinations resulted in the initiation of gametangia and their fusion. In most of these combinations the sexual interaction culminated in the production of apothecia housing asci with mostly eight wild type ascospores, i.e., full sized, fully pigmented, and striate.

Some of the combinations involving stocks *st* III and *fu* II proved exceptional and also different. The strains of *st* III when paired with a strain of opposite mating type from any of the other *st* stocks formed normal apothecia and asci. Most asci, however, produced one or more abnormal ascospores characterized by reduced size, reduced or no pigmentation, and surface warts instead of striae.

In all *fu* II x *st* combinations no asci or ascospores were produced even though all the steps leading to the initiation of apothecia appeared normal (See Bistis, 1998 for a discussion of these in *A. stercorarius*). In every case, however, the apothecia stopped growing within 24-48 h and did not form asci or ascospores. These incipient apothecia did not exceed 300 microns in diameter, whereas normal apothecia can reach diameters of 3000 microns, and more. The *fu* II stock is reproductively isolated from all six of the *st*stocks. The seven stocks represent at least two species.

Hence the results presented in this paper support the contention of Olive (1954) that the *stercorarius* - *furfuraceous*complex consists of two species. Korf and Zhuang (1991) and Perkins and Raju (1996) had accepted this view.

The stage at which the reproductive block occurs in *st* x *fu* pairings was more closely pinpointed by applying a classic technique of crushing some of these incipient apothecia under a cover glass so as to extrude their ascogonia. In all cases the ascogenous cell had budded out ascogenous hyphae, but in no case were these very extensive (See Figure 2e in Bistis, 1998). From these observations I conclude that the block to fertility in the interspecific matings occurs early in the establishment of the dikaryon in the ascogenous hyphae. A similar block may also be involved in some interspecific incompatibilities in *Neurospora* (Perkins and Raju, 1986) and in the *Ascobolus immersus* complex (Lissouba, 1960; Meinhardt *et al.*, 1984). In both instances some of the interspecific pairings yielded only small rudimentary ascocarps. These were not examined, however, to determine the cytological condition of the ascogonium. In all these cases of early arrest the block may be at internuclear recognition (Debuchy, 1999), which is a stage in the sexual cycle that requires the functioning of two mating types to establish the dikaryon. This stage is also involved in sexual isolation between species in basidiomycetes (Perkins, 1994). The two mechanisms differ, however, in that the dikaryon of euascomycetes is dependent, in contrast to the independent dikaryon of basidiomycetes.

One final point comes from the observation that in crosses between an *st*III strain with any compatible one from any of the other *st* stocks most asci contain one or more abnormal ascospores. No tests of the ability of ascospores from these asci to germinate and also to produce viable colonies were made. It is not known whether or if *st* III is on its way to becoming reproductively isolated from the six other *st* stocks. For the present, based on morphology, it is still an *A. stercorarius*. This is in sharp contrast to the situation of the *fu*II stock, where the isolation with the *st* stocks is complete.

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