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Review

Ascomycete phylogenetics: Morphology and molecules

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Mycologists have been quick to use genotypic data to examine questions arising from the field's long history of phenotypic investigation (Bruns et al., 1991; Reynolds and Taylor, 1993). In many cases, phylogenetic inference from nucleotide sequence data has been robust in fungi (Bruns et al., 1992; Nishida and Sugiyama, 1992; Berbee and Taylor, 1992; Spatafora and Blackwell, 1993), making it unnescessary to add phenotypic characters in hopes of resolving an ambiguous result. This omit affirmative result is not always the case, and there are instances where molecular characters have been unable to resolve ambiguities; e.g., are chytrids and zygomycetes each monophyletic (Bruns et al., 1992), or is *Pneumocystis* an ascomycete, a basidiomycete, or neither (Taylor et al., 1993)? In these cases, morphological characters may provide evidence that is lacking from the molecules, e.g., flagella in chytrids and their absence in zygomycetes, or asci in *Pneumocystis*.

When it comes to analyzing phenotypic and genotypic data there are two schools of thought. One argues from first principles that all data must be included in an analysis because to do less would admit bias (Kluge, 1989). The other argues that each data set should be analyzed independently, and the results compared to see if a conflict exists between the two inferred phylogenies. If there is a conflict, then the datasets should not be combined, but rather reexamined for their appropriateness (Bull et al., 1993) We have favored independent analysis followed by examination of morphological characters against the molecular phylogeny (Berbee and Taylor, 1992), but we have not conducted independent cladistic analyses of phenotypic and genotypic characters to see if the inferred phenotypic phylogeny is in conflict with the genotypic one. Using a group of fungi for which a genotypic phylogenetic analysis exists (LoBuglio et al., 1993), we conducted a phenotypic analysis using known morphological and physiological characteristics to examine the interplay of phenotype and genotype. We cannot claim to have the total evidence, but we have selected phenotypic characteristics that have been used to justify classification of these fungi.

Our example of the interplay of morphological and molecular data is provided by the classification and implied phylogenetic relationships of three *Penicillium* species that form their conidia on tightly clustered conidiophores: *P. duclauxii* Delacr., *P. clavigerum* and *P. vulpinum* (Cooke & Massee) Seifert & Samson. Conidiophores of the first two species form elongated synnemata, which have conidiophores over the entire surface, but *P. vulpinum* makes a coremium in which the conidiophores are restricted to the swollen head and are absent from the stalk. Penicilliologists use the branching pattern of the typically solitary conidiophores to classify species to subgenus, but this pattern is difficult to interpret in *P. clavigerum* and *P. vulpinum*, possibly as a result of these species having clustered conidiophores. Raper et al. (Raper et al., 1949, p. 612) commented on this problem: "···it is questionable whether a sharp line of separation can be drawn between [*Penicillium duclauxii*] and *Penicillium clavigerum*···some penicilli in *P. clavigerum* consist of a single terminal verticil of metulae and strongly suggest the Biverticillata-Symmetrica. Conversely, some penicilli in *P. duclauxii* are branched and asymmetrical, hence suggest the pattern regarded as characteristic of *P. clavigerum*."

As a result, the classification of these three species has been controversial. To attempt to resolve this controversy, we used nucleic acid sequence from the nuclear ribosomal DNA region containing the two internal transcribed spacers and 5.8S rRNA gene (ITS1-5.8S-ITS2) and mitochondrial small subunit rRNA genes (mtSrDNA) to examine the phylogenetic relationships of *Penicillium, Talaromyces* and *Eupenicillium* species, including *P. duclauxii, P. vulpinum* and *P. clavigerum* (LoBuglio et al., 1993). Here we compare these results to an analysis of the morphological characters.

Morphological characters Three groups of systematists have considered these species critically: Raper et al. (1949), Pitt (1979) and Samson et al. (1976). All agree that *P. duclauxii* makes biverticillate conidiophores, and have placed it in the subgenus *Biverticillium*. Raper and colleagues, and Samson and Colleagues did not detect biverticillate conidiophores in *P. vulpinum* (=*P. claviforme* Bainier) or *P. clavigerum*, and placed these two species in section *Asymmetrica*, subsection *Fasiculata* (=subgenus *Penicillium* Pitt). Pitt did not place as much emphasis on conidiophore branching in species with fused conidiophores and, instead, he emphasized phialide shape. In these three species, and in the subgenus *Biverticillium*, he consider the phialides to be

acuminate or acerose; this is in opposition to the subgenus *Penicillium*, where it is agreed that the phialides are ampuliform. As a result, Pitt considered *P. clavigerum* to be a synonym of *P. duclauxii*, and he classified *P. vulpinum* and *P. duclauxii* in subgenus *Biverticillium*. This debate was last visited by Stolk et al. (1990), who disputed Pitt's observation of acuminate phialides in *P. vulpinum* and *P. clavigerum*, and noted that *P. vulpinum* and *P. clavigerum* made mycotoxins, ubiquinones, and other secondary compounds typical of subgenus *Penicillium*, rather than subgenus *Biverticillium*.

Our phenotypic analysis is limited to *P. duclauxii, P. vulpinum, P. clavigerum*, one representative of the subgenus *Biverticillium* clade (*Talaromyces flavus/Penicillium dangeardii*), and two representatives of the subgenus *Penicillium* clade (*Eupenicillium ochrosalmoneum* and *E. javanicum*). Of course, the *Eupenicillium* species do not have mitosporic states in subgenus *Penicillium*, but Peterson (1993) has shown *Eupenicillium* species to be on the same clade as species in the subgenus *Penicillium* based on comparison of partial 28S rRNA gene sequence from representatives of the Trichocomaceae. So, we consider the *Eupenicillium* species to be representatives of the subgenus *Penicillium* clade.

We selected nine common morphological characters (Table 1) used to classify these fungi (Raper et al., 1949; Pitt, 1979; Samson et al., 1976). (1) Synnema form (acicular or not), (2) coremium form (clavate or not), (3) biverticillate conidiophore (present or absent), (4) terverticillate conidiophore (present or absent), (5) monoverticillate conidiophore (present or absent), (6) acerose phialide (present or absent), (7) ampuliform phialide (present or absent), (8) ratio of conidium length to width (>1.3 or <1.2), and (9) regularity of conidium chain (regular or irregular). Because we are unsure of the independence of related morphological characters, we chose presence and absence as character states; e.g., rather than use coremium or synnema as states of a single character, we treated them as separate characters, each with the states of presence or absence. We also used characters reflecting seven day's growth at various temperatures and water activity (Table 1) as described by Pitt (1979). (10) CYA at 25°C (<30 mm or >30 mm), (11) MEA at 25°C (<30 mm or >30 mm), (12) CYA at 37°C (0 mm or >15 mm), (13) G25N at 25°C (<8 mm or >8 mm), (14) CYA at 5°C (>2 mm or 0 mm). Lastly, we used the type of ubiquinone molecule (Q10(H_2) or Q9) (Kuraishi et al., 1991). In Table 1, for each character the first state given above is coded as (1) and the second as (0). We expected secondary compounds to be important to this analysis, and although each of the six species, except *E. javanicum*, have been reported to make secondary compounds (Frisvad and Samson, 1991; Samson et al., 1989; Pitt and Leistner, 1991), all were unique to each species (apomorphic) and were not included in the analysis.

Molecular characters LoBuglio et al. (1993) have used nucleotide sequence of the mtSrDNA and ITS1-5.8S-ITS2 rDNA region to analyze the relationships of *P. duclauxii, P. vulpinum*, and *P. clavigerum* along with 19 other Trichocomaceae and two outgroup species. For these taxa, there were a total of 913 bp, 132 of which were informative in the cladistic sense. Parsimony analysis of all 24 taxa showed that *P. duclauxii* was on a clade with *Talaromyces* species, and that *P. vulpinum* and *P. clavigerum* were on a clade with *Eupenicillium* species. This result was found when the nuclear and mitochondrial data were analyzed imdependently, or when they were combined. Parsimony analysis of bootstrapped data sets showed strong support for branches at the base of the clade containing *Eupenicillium* species and *P. clavigerum* and *P. vulpinum*, and at the base of the clade containing *Talaromyces* species and *P. duclauxii*.

Phylogenetic analysis Parsimony analysis of just the phenotypic characters among the six species of *Penicillium, Talaromyces* and *Eupenicillium* resulted in one unrooted most-parsimonious tree of 21 steps (Fig. 1). No matter where the root is placed, the species with clustered conidiophores can never be closer relatives to *Eupenicillium* species than *Talaromyces* species. Analysis of 1000 bootstrapped data sets shows weak support for all of the internal branches.

Parsimony analysis of combined mtSrDNA and ITS1-5.8S-ITS2 rDNA sequence for the six species, using 1000 bootstraped data sets, gave a different tree (Fig. 2) than the phenotypic data. In this tree, all of the internal branches are strongly supported, and it has the same topology as seen in the analysis of 24 species of Trichocomaceae (LoBuglio et al., 1993).

The phenotypic tree and the genotypic tree differ, but is this difference significant? Using an analysis similar to one performed by LoBuglio et al. (1993), we compared both trees against the genotypic data using maximum likelihood (DNAML in PHYLIP; Felsenstein, 1991).

Table 1.

Taxa/Characters	1[1]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
P. duclauxii	1	0	1	0	0	1	0	1	0	1	1	1	0	1	1
P. vulpinum	0	1	0	1	0	1	1	1	1	0	1	1	0	1	0
P. clavigerum	1	0	1	1	0	1	0	1	1	0	1	1	0	1	0
T. flavus	N[2]	N	1	0	0	1	0	1	0	1	0	0	1	0	1
E. ochrosalmoneum	N	N	0	0	1	0	1	0	0	1	0	0	1	0	0
E. javanicum	N	N	0	0	1	0	1	0	1	0	0	0	0	0	0

[1] Characters and character states described in text. [2] N represents character not applicable.

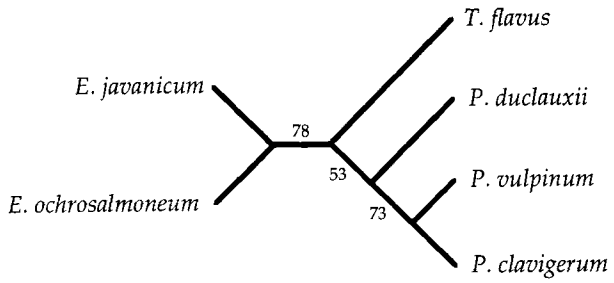

Fig. 1. Tree from parsimony analysis of phenotypic characters given in Table 1. Numbers adjacent to internal branches represent percentage of trees having the branch from 1000 trees based on bootstrapped data sets (Felsenstein, 1985) using the heuristic search option in PAUP 3.1.1 (Swofford, 1991).

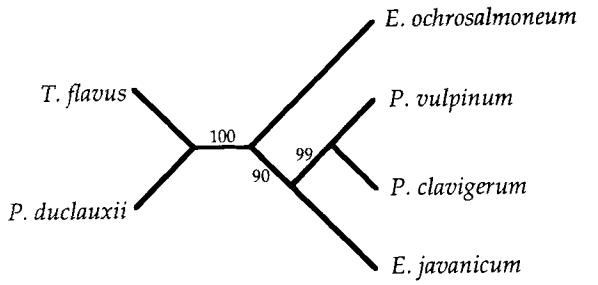

Fig. 2. Tree from parsimony analysis of genotypic characters (ITS, 5.8S and mt SSU rDNA) given in LoBuglio et al. (1993). Numbers adjacent to internal branches represent the percentage of trees having the branch from 1000 trees based on bootstrapped data sets (Felsenstein, 1985) using the heuristic search option in PAUP 3.1.1 (Swofford, 1991).

As found by LoBuglio and colleagues, given the genotypic tree, the data were significantly more likely than they were given the phenotypic tree (by nearly nine standard deviations; log likelihood given the genotypic tree -1775, log likelihood given the phenotypic tree -2027, S. D. 28). It would be wonderful if the same type of analysis could be performed with the phenotypic data and both trees, but there is no way of assigning probabilities to changes in phenotypic characters. Instead, we used MacClade (Maddison and Maddison, 1992) to compare the number of character state changes on the genotypic (23 steps) and phenotypic (21 steps) trees. The genotypic tree was only two steps longer than the phenotypic tree. Although the genotypic data are unlikely given the phenotypic tree, the phenotypic data do not seem unlikely given the genotypic tree because the phenotypic data can be accommodated on the molecular tree with only two additional steps. Therefore, it appears that there is no conflict between the two types of data. In this case, there would seem to be no harm in analyzing the combined data sets, but would there be a benefit? Parsimony analysis of the combined phenotypic and sequence data, again with 1000 bootstrapped data sets, gave a tree with the same topology as the molecular data alone (Fig. 2), but with reduced support for two of the internal branches (from 99 to 94, and from 90 to 62). It is, therefore, hard to argue that a substantial benefit accrued from the combined analysis.

Although genotype appears most valuable in determining phylogenetic relationships, the importance of phenotypic characters in fungal life history and adaptation cannot be overemphasized. Analyzing the evolution of phenotypic traits with the knowledge gained from a genotypic data set is one of the most exciting possibilities gained from molecular studies. When the phenotypic, genotypic and combined trees are compared with phylogenies inferred from historical classification, it is apparent that the phenotypic analysis is consistent with Pitt's classification (Pitt, 1979), while the molecular and combined analyses are consistent with that proposed by Raper et al. (1949) and endorsed by Samson et al. (1976). Statistically, support for the molecular tree is strongest, and that for the phenotypic tree is weakest.

If the phenotypic characters are mapped to the molecular or combined tree (Fig. 2), seven of the 15 characters show homoplasy. Two of these involve morphological characters whose interpretation is problematic (i.e., conidiophore branching pattern and phialide shape), while the other homoplasious characters may simply not be useful in distinguishing subgenera (i.e., conidium shape and growth at extreme temperatures and water activity). Of the eight non-homoplasious characters, three characters were particularly valuable in determining relationships of the Penicillia with clustered condiophores: lack of a biverticillate conidiophore as determined by Raper et al. (1949), ampuliform conidia as determined by Samson et al. (1976), and the type of ubiquinone (Kuraish et al., 1991).

In the case of these *Penicillium* species, analyzing the phenotypic and genotypic data separately or in combination gave the same final result because there was no significant conflict between the two datasets. This situation should be very common with fungi because there are few informative phenotypic characters compared to a far larger number of demonstrably and potentially informative molecular characters. Although the phenotypic characters may be obscured by the genotypic characters in a combined analysis, they can be brought to prominence when their evolution is examined against the genotypic phylogeny.

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