# Multilocus sequence analysis of *Penicillium* and *Eupenicillium* species

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*Summary* Taxonomy of Hyphomycetes has always been a challenging problem, with experts viewing species in different ways and modifying the taxonomy of groups to reflect their best evaluation of species limits and concepts. The advent of phylogenetic analysis, relatively easy DNA sequencing techniques and PCR has provided an opportunity for mycology to move from a strictly morphological analysis of species to phylogenetic analysis of DNA sequences. Phylogenetic theory dictates that data from different loci will produce congruent or at least non-contradictory evolutionary histories of a clonal lineage. Tests of tree congruence such as the index of association can show whether lineages are clonal, and has revealed that some species long thought to be clonal are cryptically recombining. Genealogical concordance phylogenetic species recognition allows unambiguous identification of species boundaries.

*Key words* Phylogenetics, Concordance, Hyphomycetes

## Introduction and history

Phylogeny is the study of patterns of organismal descent deduced from comparative analysis of homologous features or attributes. Hennig [19] proposed a system for phylogenetic systematics with the primary requirements that members of a species be related by descent, that character evolution be polarized by comparison of the ingroup (study group) with homologous characters in the outgroup (most closely related species not in the study group) and that species be recognized on the basis of synapomorphic (shared-derived) characters rather than plesiomorphic (ancestral) characters.

Phylogeny of evolutionary lineages historically has been studied by examining fossils in datable rock strata. This is a reliable way to estimate times of divergence, but is limited by insufficient sampling of fossil beds [62]. Fungi have a fossil record that is incomplete and possibly will remain that way because of the difficulties of recovering these fossils and the failure of fossilization in many soft tissued species [51].

Lacking a good fossil record, mycologists have turned to molecular methods to study fungal phylogeny. Early sequencing studies were mostly based on ribosomal (r) RNA molecules. rRNA molecules are present in cellular organisms as a large percentage of the total nucleic acids. Crude preparations of nucleic acids can be extracted and the rRNA can be sequenced directly. Studies based on 18S rRNA sequences answered many long-standing evolutionary questions and led to major revisions of the systematics of bacteria [73, 74] and the fungi [2, 4, 5, 34, 71]. The first sequence based phylogenetic studies in the Penicillia and Aspergilli used 28S rRNA sequences [10, 30].

In the past 20 years some major theoretical and technical advances have made the study of DNA sequences tractable. We can easily amplify specific loci using PCR [52] and sequence the DNA from those amplified fragments [32, 56]. With automated DNA sequencing equipment using fluorescent dye technology we are free of the hazard of radioactive tracers used in the past. Because DNA sequencing has become an accessible technology for many mycologists, the challenge is to carefully collect data and interpret it in an appropriate manner, melding organismal knowledge with phylogenetic knowledge [70].

## Molecular phylogenetics of fungi

### *Sequencing artifacts*

Practical DNA sequencing methods introduced in 1979 [32, 56] no longer have significant technical problems, but low quality sequences still appear in GenBank. Primarily these problems are related to the purity and quality of reagents, the amplified DNA, and somewhat less frequently to the conditions of electrophoresis. Oligonucleotide primers used in PCR define the ends of the DNA fragment being amplified and must be sufficiently specific (18-20 base length) that only the targeted gene is amplified. Lower specificity may allow two or more loci to co-amplify from a single primer pair. Cross contamination of cultures, DNAs and reagents used in PCR [23] can also cause amplification of a mixed sequence DNA fragment or fragments. Sequences from mixed amplicons are unreadable.

DNA sequences from pure amplicons can be incorrectly read if the DNA strands are not completely denatured during electrophoresis. In high mol% G+C DNAs such as *Penicillium* species ITS region, one must be very careful

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to avoid partial renaturation during electrophoresis, which causes compression artifacts. When compressions occur, more than one fragment migrates in the gel with the same apparent molecular weight. Instead of a regular ladder of DNA fragments each one base longer than the previous one, there will be steps missing from the DNA ladder, and some steps will be doubled because two different DNA fragments are co-migrating. The total number of bases read will be less than the number actually present in the amplified fragment. The co-migrating fragments will both be detected at the same base position making the true sequence uncertain. Such positions are reported as Ns. Some studies using ITS or 28S rRNA sequences from *Aspergillus* and *Penicillium* species [15, 30, 38, 68] sometimes include sequences that appear to be compressed. Including an isolate of known sequence in a DNA sequencing project provides a useful positive control. Using sequences containing unreadable base positions as the basis for phylogenetic inference is problematic unless contamination and compression artifacts can be excluded as causes of the Ns.

DNA sequences in protein coding genes are translated into amino acids. Many DNA analysis programs predict amino acid sequences from the DNA sequence. If the DNA sequences from protein coding genes cannot be translated into proteins, the DNA fragment either represents a pseudogene or the primary sequence is flawed.

Some DNA contains repetitive sequence elements such as poly A (10-20 consecutive adenosine residues). The sequence read after poly A regions is usually unclear, possibly due to infidelity during amplification or sequencing reactions. Sequences are normally clear and readable from either end until the poly A region. Designing and using sequencing primers close to the poly A region will usually allow the sequence to be read. DNA regions containing alternating runs of poly G and poly C are likely to self-anneal during sequencing, forming hairpin double stranded loops in the DNA that terminate polymerase activity. In those cases the sequence stops or is very weak after the hairpin regions. Raising the temperature for the elongation reaction by 2-3 °C can sometimes overcome this type of problem. Capillary sequencers that use premixed gel polymers and denaturants, and that have very precise temperature control during electrophoresis generally prevent compression artifacts. Older sequencers where gels and denaturants are freshly made in the lab, and which have less sensitive temperature control during electrophoresis must be monitored closely to prevent compressions.

### *Analytical techniques*

Alignment of DNA sequences is performed using programs such as CLUSTAL [66] followed by visual optimization of the alignment. Visual examination is crucial because CLUSTAL does not always give proper alignments when the sequences have significantly different lengths. ITS sequences vary greatly in length, and some protein loci have different lengths due to variable numbers of introns. Phylogenetic analysis of aligned sequences can be performed using program packages for phylogenetic analysis. These include PAUP* [64], PHYLIP, MEGA and many others [http://evolution.genetics.washington.edu/phylip.html]. There are three main analytical approaches based on genetic distance, parsimony or likelihood. The neighbor-joining technique of tree formation is computationally quick and the method for computing genetic distance can be chosen to fit the evolutionary model. An appropriate evolutionary model can be determined from the dataset using MODELTEST [48]. Models of DNA evolution are discussed by Nei [35] and Nei and Kumar [36]. Parsimony analysis also requires relatively little computational power and has the implicit assumption that only synapomorphies are used in determining the topology of the tree. Likelihood methods are computationally intensive and impractical for tree searching in large datasets. However likelihood programs are very useful for testing alternative tree topology hypotheses [27] and operate with reasonable speed under these conditions. Statistical support for branches in the tree are most commonly calculated using the bootstrap method and can also be assessed with Bayesian analysis [21].

### *Choice of locus*

The advent of PCR gene amplification made more choices available than just the rRNA. ITS and IGS regions of the rRNA repeat unit have been used to address species level questions in fungi. ß-tubulin [18] is readily amplified and sequenced in many fungi. The portion of ß-tubulin identified as the BT2 amplicon includes amino acid coding exons whose sequences tend to be conserved and noncoding intron segments that seemingly are free from selective pressure and thus approximate the expected random changes of genetic drift. Calmodulin has been widely used in phylogenetic studies of fungi [11,37] and the primers used by Peterson et al. [46] amplify nearly the entire calmodulin gene. Translation elongation factor 1-alpha (ef1-α) has been amplified and sequenced in *Penicillium* species, but interpretation of sequence data from ef1-α is complicated by the variable number and location of introns in the 5' half of the molecule. RNA polymerase [28] is being used in the all fungal tree of life (AFTOL) project [31] and appears to be a useful locus for species level studies in *Penicillium* (unpublished). A number of mitochondrial loci have also been used including mitochondrial small subunit rDNA [29]. As whole fungal genomes are completed it will become even easier to select additional loci and design suitable primers for amplification and sequencing.

### *Taxon sampling*

In addition to choosing DNA loci that have appropriate levels of variation for the questions being asked, incomplete taxon sampling of the ingroup species can confound analysis. As an example, Peterson [39] published a phylogenetic tree containing ca. 120 *Penicillium* and *Eupenicillium* species. Lineages supported at the 95% bootstrap level have subsequently proved to be stable; species from less strongly supported groups display instability, appearing on different branches of the tree as taxa are added or removed. *Penicillium charlesii* was portrayed in the tree [39] as having an undetermined basal position among the *Penicillium* species. Peterson et al. [41] could not firmly place this species in the larger tree, but subsequently [46] were able to satisfactorily establish its placement in the tree along with *Penicillium coffeae, Penicillium indicum, Penicillium phoeniceum, Penicillium chermesinum* and *Penicillium fellutanum*. Finding the true position of *P. charlesii* among *Penicillium* spp. depended on the addition to the dataset of sequences from newly described species and species previously believed to be synonyms. As some of the species from the dataset that resolves the position of *P. charlesii* are removed, this species once again becomes unstable in the larger *Penicillium* tree moving from branch to branch.

Some lineages in the *Eupenicillium* clade are poorly sampled. Based on the large genetic distance between some species in the tree, it is reasonable to predict that

there are many undescribed species of *Penicillium*. Samson [53] listed 25 species of *Penicillium* and *Eupenicillium* newly described between 1992 and 1999. The current literature suggests that the rate of new species descriptions has not slowed since 1999. Because we are still discovering and describing the diversity in *Penicillium* and because some species are extinct, complete taxon sampling in each clade is not possible and the position of some species in the tree will not be fully resolved.

In addition to complete taxon sampling, it is highly desirable to sample multiple isolates of each species. When investigating intraspecific questions instead of generic phylogeny, populations must be sampled intensively. Twenty or more isolates from each population provide the basis for strong statistical inference. While gene sequencing is the most generally useful DNA technique, more rapid techniques such as microsatellite analysis [8,12,13,20], inter-simple sequence repeats (ISSR) [67], multilocus AFLP [61] and other techniques can provide less costly ways to detect polymorphisms at additional loci and answer specific questions quickly.

### *Species concepts*

For many years the morphological species concept was the only species concept available. Each generation of mycologist made the best possible interpretation of the data available, but even so, experts came to different conclusions about where species boundaries occurred [47,49,50] and which taxa to include in the various genera. Other species concepts used in mycology include the biological species concepts (BSC) based on potential or real gene flow between populations, the phylogenetic species concept (PSC) with a primary requirement of monophyly [3,33], and chemical taxonomy based on detailed knowledge of the secondary metabolites produced [14,16].

The literature on *Penicillium* systematics includes many studies that used single locus trees for phylogenetic inference [24,39,42,44,54,57,59,69]. These studies reveal the relationships of species in *Penicillium* and genetic distance arguments are invoked to support separation of the isolates into species. This interpretation relies on a molecular clock and the very regular accumulation of sequence variation. It also relies on the idea that sequence variation at some definable level is representative of species distinctions. However, sufficient questions have arisen about uniformity of the molecular clock that defining species on the basis of genetic distance must be viewed as a hypothesis that needs to be tested.

Avise and Ball [1] advocated the use of genealogical concordance in phylogenetic studies in order to preclude the possibility of incorrect phylogenies caused by the use of genes that for one reason or another might not be representative of the evolutionary history of the organism [e.g., 22]. Koufopanou et al. [26] reasoned that in clonally reproducing organisms (e.g., Hyphomycetes) data sets from unlinked loci should generate trees that are fully concordant or at least non-contradictory. Instead of fully concordant trees from the different loci, the patterns of polymorphisms they found in the putative Hyphomycete *Coccidioides immitis* genes studied were within expectations for recombining species. Other studies [6] proved that *C. immitis* is undergoing genetic recombination, even though a teleomorph has never been observed. Of course, this may relate to parasexuality.

Geiser et al. [17] showed that *Aspergillus flavus* contained two distinct and genetically recombining cryptic species through analysis of polymorphisms at multiple unlinked genes. Peterson et al. [43] demonstrated the genetic isolation of *Aspergillus bombycis* through the application of multilocus DNA sequence analysis. Studies using multilocus data to define species limits in *Penicillium* are being published [40,41,46,67]. From examining the results of the studies mentioned, it is clear that the molecular clock is not perfectly regular in the time frame of sibling species. Species defined phylogenetically using multilocus sequence data may have perfectly uniform DNA sequences in the ITS, or other loci, or they may have small levels of variation. Unless the differences in ITS sequence are large it is not clear from genetic distance arguments that isolates represent different species.

Taylor et al. [65] argued that the evolutionary species concept (ESC) [60, 72] is an adequate species definition for use in fungi. The ESC describes what a species is without reference to any of the mechanisms [25] by which species arose. They argue that the problem facing taxonomists is not in species concepts, but in how we recognize the limits of species. Most Hyphomycetes species display patterns of polymorphisms indicative of current or recent genetic recombination. For these species the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) [65] system recognizes genetic isolation as the species boundary. GCPSR was tested against biological species recognition (mating tests) in *Neurospora* [9]. The multilocus phylogenetic analysis recognized each of the species that could be diagnosed using mating tests, and also recognized three additional species. Although there have been suggestions that some *Neurospora* species hybridize, the multi-locus DNA sequence data did not find any support for that hypothesis.

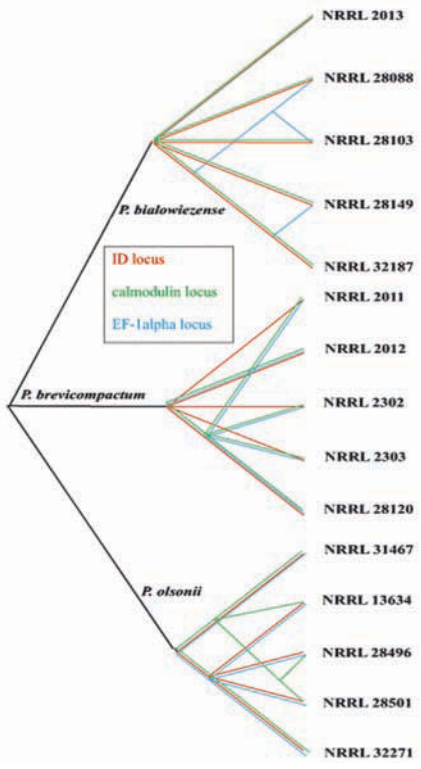


Figure 1. Phylogenetic tree composed of data [40] from the ID locus (in red), the calmodulin locus (in green) and the translation elongation factor 1-alpha (in blue) from isolates in the *P. brevicompactum* clade. The concordant lines from the species to the most recent common ancestor signify that the data from the three loci are not discordant. The differing patterns of descent at the tips of the branches (discordance), signified by the crossing of tree branches based on different loci, suggest strongly that genetic recombination is or recently has been occurring among the isolates. Species boundaries are drawn at the point where concordance among the different genes is lost.

GCPSR was also applied to *Penicillium brevicompactum* and some closely related species [40]. When data from the three loci are plotted as layers (Figure 1) on a single diagram, one can quickly recognize the species boundaries. Branches are concordant, or in this case, not discordant, from the most recent common ancestor up to the tip area of the tree. The branching order of the three species is not specified in this tree because the data lack statistical support for which two of the three species are siblings. In the tree tip areas, the patterns of descent based on each of the three loci are discordant (Figure 1). This very strongly implies that genetic rearrangements are occurring, or have occurred in the recent past. When isolates are sharing the same gene pool, as the isolates in each of these three species appear to be doing, the GCPSR interpretation of the data is that they are isolates of a single species.

Examining increasingly polymorphic genes will lead to the description of more and more species when using a single locus to define species. This possibility is seen in *C. immitis* microsatellite loci where there is sub specific structure [12]. When the multilocus approach is followed, even with highly polymorphic loci, the limits of species, as defined by gene flow, remain the same. GCPSR could be confounded by balancing selection of the loci used. The discipline of population genetics has devised an array of statistical tests to recognize selection [35,36] and the use of such tests is necessary to insure that the data are appropriate for the analytical model. Some of the limitations of particular tests used in phylogenetic analysis are summarized by Sanderson and Shaffer [55] and the testing of new analytical techniques is ongoing and reported regularly [7,27,62].

Once the phylogeny of *Penicillium* is resolved by the use of multilocus concordance species recognition and the species are known, it will be possible to distinguish synapomorphies from plesiomorphies and apply the principals of phylogenetic systematics to any of the data sets we have. Morphological, chemical, physiological or DNA sequence data [37,58] can be used to identify species in the different research environments where those techniques are most practical.

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