Progress towards a phylogenetic classification of *Fusarium*

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A DNA sequence database is being developed for all species of *Fusarium* that will be used to construct a phylogenetically-based classification and develop molecular tools for the rapid and accurate identification of unknown isolates. Multiple genetic loci are being sequenced to generate independent phylogenetic hypotheses in order to identify monophyletic taxa and resolve sister group relationships. Deficiencies in morphological and biological species concepts are illustrated by selected examples within two paraphyletic sections, *Liseola* and *Martiella*. Both groups contain polytypic species (e.g., *F. verticillioides*, synonym = *F. moniliforme* and *Nectria haematococca/F. solani*) for which a phylogenetic species concept is being developed. Phylogenetic reconstruction from the molecular data indicates that *Fusarium*, *Nectria* and subgenus *Dialonectria* are paraphyletic as currently defined. A proposal for elimination of the paraphyly of these taxa is presented.

Keywords: molecular biology, phylogeny, taxonomy.

Fusarium is a large cosmopolitan genus of pleoanamorphic hyphomycetes whose members are responsible for a wide range of plant diseases (Farr & al., 1989), mycotoxicoses, and mycotic infections of humans and other animals (Nelson & al., 1994). Collectively the fusaria represent the most important phytopathogen and mycotoxigenic genus of filamentous fungi (Marasas & al., 1984). In addition to producing a wide range of toxins, including trichothecenes and fumonisins, the fusaria are noted for their production of other secondary metabolites such as gibberellin plant growth hormones, named after *Gibberella fujikuroi* from which they were first discovered.

Fusarium is one of the most challenging genera for morphological systematists (Gams & Nirenberg, 1989). Traditional classification and identification schemes are based exclusively on a morphological species concept derived from cultural characteristics of single-spore isolates grown on special media, shared morphological traits of the anamorph, host range, and, to a lesser extent, teleomorph micromorphology (Booth, 1971). Given the paucity and plasticity of phenotypic traits, it is not surprising that conflicting morphological species con-

cepts are employed in taxonomic treatments of this genus (Booth, 1971; Gerlach & Nirenberg, 1982; Nelson & al., 1983). For this reason, the systematics of *Fusarium* remain controversial and confusing, especially if more than one taxonomic treatment is consulted. Gerlach and Nirenberg's system (1982) is the most differentiated, including 73 species and 26 varieties; Booth (1971) recognizes 44 species and 7 varieties and Nelson & al. (1983) only 30 species.

Given the wide divergence in taxonomic opinions reflected in the competing morphologically-based systems of classification, a molecular systematic approach based on discrete DNA sequence data offers considerable promise in the establishment of an objective, phylogenetically-based system of classification for *Fusarium* and its teleomorphs (Bruns & al., 1991). Previous molecular systematic studies on the genus have demonstrated the utility of partial nuclear large subunit (LSU) 28S rDNA sequence comparisons for phylogenetic analysis (Guadet & al., 1989; Peterson & Logrieco, 1991; O'Donnell, 1993); taxon sampling in these studies, however, was limited and the level of resolution obtained using LSU 28S rDNA sequence data is insufficient, for example, to distinguish the biological species within section *Liseola* and to apply a phylogenetic species concept (O'Donnell & Cigelnik, 1995b; O'Donnell, unpubl.).

With the recent advent of polymerase chain reaction (PCR) methodology (Mullis & Faloona, 1987) and non-radioactive, automatic DNA sequencing, it is now possible to amplify and sequence specific regions of the genome from a large number of taxa to test the morphologically-based taxonomic hypotheses developed for the fusaria. Towards this end, we are developing a DNA sequence database from the following four loci for all species of *Fusarium*: beta tubulin gene introns/exons, mitochondrial small subunit (mtSSU) rDNA, nuclear large subunit (LSU) rDNA, and nuclear rDNA internal transcribed spacer (ITS) region. Specific questions being addressed in these cladistic studies include:

1. What is *Fusarium*?
2. Are *Nectria*, *Gibberella*, and *Fusarium* monophyletic?
3. Are the sections natural?
4. Can a phylogenetic species concept (PSC) be applied, and to what extend is it congruent with morphological and biological species concepts?
5. Can sister group relationships of all taxa be resolved?
6. How can the DNA database best be used to design molecular tools for the detection, and rapid and accurate identification of all species of *Fusarium*?

This paper is a progress report of efforts towards these objectives.

Material and methods

Microbiology

Isolates are stored cryogenically at –175 C or by lyophilization in the ARS Culture Collection (NRRL, NCAUR, Peoria, IL). Most strains were obtained from the American Type Culture Collection (ATCC), Biologische Bundesanstalt (BBA), Centraalbureau voor Schimmelcultures (CBS), Fusarium Research Center (FRC), International Mycological Institute (IMI), and Institute for Fermentation (IFO).

DNA extraction from mycelium

Mycelium for DNA extraction is grown in YM broth culture (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 2% dextrose), harvested and lyophilized overnight as described previously (O'Donnell, 1992). DNA is extracted using a modification of the SDS protocol of Raeder & Broda (1985). Lyophilized mycelium (~ 50 mg) is pulverized in a 1.5 mL microfuge tube with a pipet tip, resuspended in 600 µL extraction buffer (200 mM Tris-HCl pH 8.3, 200 mM NaCl, 25 mM EDTA pH 8.0, and 0.5% SDS) and extracted with phenol:chloroform (7:3) and chloroform. DNA is precipitated from the aqueous layer by the addition of 0.54 volumes of isopropanol and pelleted for 1 min in a microfuge at 10,000 rpm. The pellet is washed gently with 70% ethanol, resuspended in 100 µL of TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and dissolved by incubating at 55 C for 1 h-to-overnight. Dilute DNA samples for PCR are prepared by adding 4 µL of the total genomic DNA stocks to 1 mL TE/10 buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0).

DNA extraction from herbarium specimens

The following protocol was developed for extraction of DNA from herbarium specimens (Bruns & al., 1990). Prior to removal of a small sample for extraction, the specimen is examined under a dissecting microscope and any contaminating debris is removed with a water-coloring brush. If possible, the sample is macerated in a 1.5 mL Eppendorf tube and then is extracted as described above and processed through the chloroform extraction step. The aqueous DNA-containing soup is treated with GeneClean II (Bio 101; LaJolla, CA) according to the manufacturer's recommendation. DNA is eluted from the glass milk with 400 µL ddH_2O, yielding the fraction that serves as a template for PCR. Because only microscopic amounts of type specimens are usually available for extraction, and other contaminating

fungi are often present, PCR products frequently need to be cloned in order to obtain readable sequence data. For cloning PCR fragments, we use the Prime PCR Cloner Kit (5 Prime → 3 Prime, Inc.; Boulder, CO). Recombinants are subjected to a bacterial DNA miniprep and the cloned insert is amplified out of the vector and sequenced directly with the Applied Biosystems (Foster City, CA) non-radioactive Taq dyedeoxy terminator cycle sequencing kit. Because the cloned DNA may not be a *Fusarium* or the clones may represent several fusaria, it is desirable to pick several recombinants and, if possible, use independent data to guide selection of the correct cloned sequence.

PCR amplification and DNA sequencing

Locations of the PCR and sequencing primers are indicated in Fig. 1 (White & al., 1990). Primer sequences are listed in Tab. 1 and in O'Donnell & Cigelnik (1995b). To facilitate sequencing DNA templates completely on both strands, internal sequencing primers

Tab. 1. – Primer sequences. For details see text in material and methods.

nuclear large subunit [LSU] 28S rDNA	
	NL1- GCATATCAATAAGCGGAGGAAAAG
	NL2- CTCTCTTTTCAATAAGCGGAGGAAAAG
	NL3- AGATGAAAAGAACTTTGAAAAGAGAG
	NL4- GGTCCGTGTTTCAAGACGG
mitochondrial small subunit [mtSSU] rDNA	
	MS1- CAGCAGTCAAGAATATTAGTCAATG
	MS11- GCAGTACTTGAGGAGGAGAG
	MS21- CTCTCCTCCTCAAGTACTGC
	MS2- GCGGATTATCGAATTAAATAAC
internal transcribed spacer [ITS]	
	ITS2- GCTGCGTTCTTCATCGATGC
	ITS3- GCATCGATGAAGAACGCAGC
	ITS4- TCCTCCGCTTATTGATATGC
	ITS5- GGAAGTAAAAGTCGTAACAAGG
nuclear small subunit [SSU] 18S rDNA	
	F11- CTTCCCTATCAACTTTCGATG
	F21- CATCGAAAGTTGATAGGGAAG
	F31- CGTAATGATTAATAGGGACAG
	F41- CTGTCCCTATTAATCATTACG
intergenic spacer [IGS]	
	NL25- CTGAACGCCTCTAAGTCAG
	CNS1- GAGACAAGCATATGACTAC

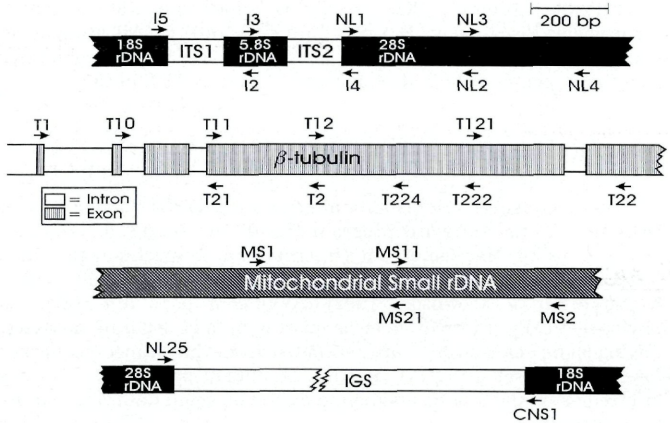

Fig. 1. – Maps showing locations of PCR and sequencing primers. Coding and noncoding regions are indicated. I and ITS = internal transcribed spacer region; IGS = intergenic spacer region. The IGS region is length variable.

were designed for the mtSSU rDNA, LSU 28S rDNA (O'Donnell, 1992), beta tubulin gene/introns (O'Donnell & Cigelnik, 1995b), and nuclear small subunit (SSU) 18S rDNA (see White & al. 1990 for PCR map). Internal sequencing primers designed for the nuclear small subunit (SSU) 18S rDNA (Tab. 1) are located between NS1 and NS2 (F11 and its reverse complement F21) and NS3 and NS41 (F31 and its reverse complement F41). In addition, a primer pair was designed and tested that amplifies the intergenic spacer region (IGS) of the nuclear rDNA (Appel & Gordon, 1995).

Following PCR amplification, primers and deoxynucleotide triphosphates are removed with GeneClean II (Bio 101; La Jolla, CA) and sequenced completely on both strands with the Applied Biosystems (Foster City, CA) *Taq* dyedeoxy terminator cycle sequencing kit in a Perkin Elmer thermal cycler programmed using maximum ramp times as follows: 25 cycles at 96 C for 30 sec, 50 to 60 C for 4 min and 15 sec, followed by a 4 C soak. Sequencing reactions are extracted once with about 50 µL of chloroform to remove the mineral oil overlay and once with an equal volume of phenol:chloroform:water (68:14:18) to remove most of the excess primers and unincorporated dyedeoxy terminators. Samples are further purified by gel filtration through 2 mL spin columns (5 Prime

→ 3 Prime; Boulder, CO) containing super-fine G50 sephadex (Pharmacia; Piscataway, NJ) equlibrated in ddH_2O. All sequencing reactions are run on an Applied Biosystems 373A DNA sequencer using a 6% gel mix (BioRad; Richmond, CA) in 1x TBE buffer.

Phylogenetic analysis

DNA sequences are visually aligned with QEdit Version 2.15, a DOS text editor software program (SemWare; Marietta, GA) and analysed using MacVector 3.5 (International Biotechnologies Inc.; New Haven, CT), PAUP 3.1.1 (Swofford, 1993), and MacClade Version 3 (Maddison & Maddison, 1992). Alignment gaps are coded as phylogenetically informative or as missing data in separate analyses. Phylogenetic relationships are estimated from the aligned sequences for each data set using PAUP 3.1.1. Maximum parsimony trees are inferred using the branch and bound option on small data sets and the heuristic search option with 1000 random addition of sequences for sets containing 20 or more taxa. Two measures of support for the phylogenetic groupings are taken: bootstrap analysis (Felsenstein, 1985) using 200–1000 replications (Hillis & Bull, 1993) with random addition input order of sequences during each heuristic search, and decay indices calculated up to 15 steps (Hibbett & al., 1995). All parsimony trees are rooted by the outgroup method. Neighbor-joining distance analyses (Tsai & al., 1994) are also performed.

Results and discussion

Fusarium DNA sequence database (NRRL)

The DNA sequence database we are developing for all species of *Fusarium* is designed to serve a dual purpose: 1. it will provide a wealth of discrete characters that will be used to reconstruct the phylogeny of *Fusarium*, to develop a natural system of classification based on cladistics (O'Donnell & Cigelnik, 1995a), as well as provide a theoretical framework for investigating the multifaceted evolutionary history of *Fusarium*, and for developing a phylogenetic species concept (PSC), and 2. the same discrete DNA nucleotide data will be used to design molecular tools such as RFLP maps, taxon-specific PCR primers, and species-specific oligonucleotides (Lee & al., 1993; Bruns & Gardes, 1993; Gardes & Bruns, 1993) so that unknown isolates can be identified rapidly and accurately.

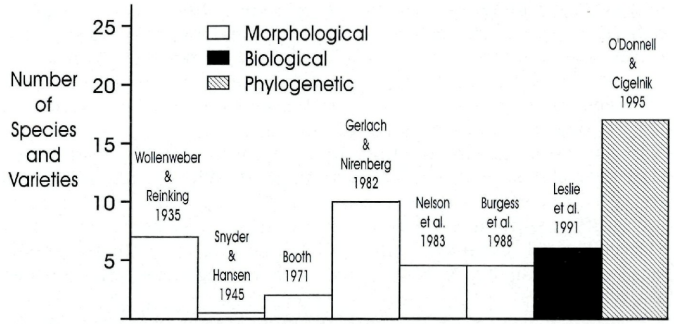

Fig. 2. – Number of species and varieties and species concepts applied to section *Liseola* of *Fusarium*.

Species concepts and phylogenetic classification

Morphological, biological, and phylogenetic species concepts have been applied within *Fusarium*. Current classifications schemes (Booth, 1971; Gerlach & Nirenberg, 1982; Nelson & al., 1983; Burgess & al., 1988) are based exclusively on a morphological species concept (MSC). Morpho-species typically are defined on the basis of a few distinctive morphological features; however, the subjective nature of this concept is punctuated by conflicts in existing taxonomic treatments of the genus.

A biological species concept (BSC) has been applied to six species within the *Nectria haematococca*-complex (Matuo & Snyder, 1973) and the *Gibberella fujikuroi*-complex (Leslie, 1989); however, most of this information has not been integrated into taxonomic schemes. While the BSC is useful for characterizing population level questions relating to gene flow and reproductive barriers, it has serious limitations for species level systematics and as a tool for identification of unknown isolates. Most notably, the concept has been developed for only a few species, mating experiments take about one month to complete, many strain fail to mate with standard testers, and negative data are equivocal.

Because we are interested in identifying sister group relationships among all of the taxa and classifying only monophyletic or natural taxa based on genealogical relationships (Vilgalys & Hibbett, 1993), we are developing a phylogenetic species concept (PSC) (Donoghue, 1985) for *Fusarium*. These species are defined as the smallest group of populations for which a unique profile of character states, including autapomorphies and synapomorphies, are fixed

within the populations (Nixon & Wheeler, 1990). Such species-defining derived characters or apomorphies form the basis of a cladistic analysis that we are extending to all species of *Fusarium*. We are interested in classifying cladistically because the genealogical relationships expressed in the classification reflects what occurs in nature. Because cladistics attempts to identify monophyletic taxa, to express sister group relationships explicitly, and polarize the character states using the outgroup method (Wiley & al., 1991), the classification can be used to make predictions about mating compatability, evolution of secondary metabolites such as mycotoxins and morphological character states (LoBuglio & al., 1993), phylogeography (Vilgalys & Sun, 1994), and phylogenetic relationships among the taxa.

How congruent are the three different species concepts? To the extent that the BSC has been applied (Matuo & Snyder, 1973; Leslie, 1989), we have found perfect congruence between the BSC and the PSC (O'Donnell & Cigelnik, 1995b; O'Donnell & Gray, 1995), indicating that both concepts represent fundamental taxonomic units. Unfortunately, the MSC is incongruent with the BSC as applied to sections *Liseola* and *Martiella* of *Fusarium* (Figs. 2 and 3). For example, Nelson & al. (1983) used a Snyder & Hansen (1941) polytypic species concept for *Nectria haematococca/Fusarium solani*, ignoring the mating studies of Matuo & Snyder (1973). Similarly, within *Liseola*, a synonym of *F. verticillioides*, *F. moniliforme*, was applied to three separate biological species or mating populations (MP) (*F. verticillioides*, MP-A; *F. fujikuroi*, MP-C; and *F. sp.*, MP-F) by Nelson & al. (1983) and *F. subglutinans* was applied to two other species (*F. sacchari* = MP-B and *F. subglutinans* = MP-E). Also, Gerlach & Nirenberg's (1982) varieties within this section are all distinct species in a biological and phylogenetic sense (*F. sacchari* var. *sacchari*, MP-B; *F. sacchari* var. *subglutinans*, MP-E = *F. subglutinans*; *F. sacchari* var. *elongatum* = *Fusarium* sp. nov.; and *F. proliferatum* var. *minus* = *Fusarium* sp. nov.). Using a PCS and phylogenetic reconstruction of DNA sequences from multiple loci in which most of the phylogenetic signal was derived from the beta tubulin gene and the mitochondrial small subunit rDNA (Fig. 1), we have identified 17 species within the *Liseola* lineage or *Gibberella fujikuroi*-complex and about half of these species form chlamydospores (O'Donnell & Cigelnik, 1995b). Section *Liseola*, however, is paraphyletic by definition since it artificially excludes chlamydospore-forming species (Wollenweber & al., 1925; Wollenweber & Reinking, 1935). Based on the results presented at the *Fusarium* meeting in Martina Franca (Summerell & al., 1995; Nirenberg & Aoki, 1995), additional morphologically and/or phylogenetically distinct species will be recovered in future surveys of biogeographically and ecologically

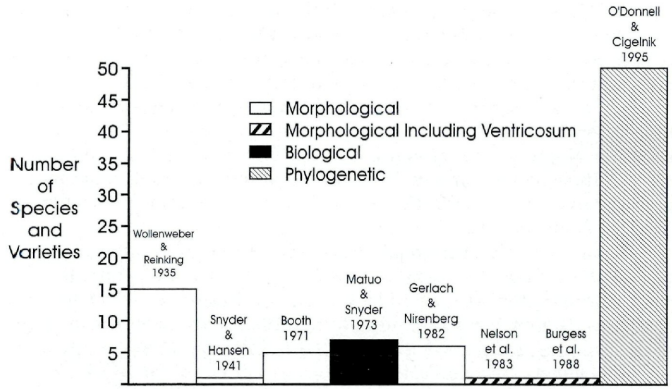

Fig. 3. – Number of species and varieties and species concepts applied to section *Martiella* of *Fusarium*.

interesting regions, further complicating the application of a MSC within this species complex. Currently, we are using the species phylogenetic hypothesis to extend the BSC within this economically important group.

Snyder & Hansen's (1941, 1945, 1953) polytypic MSC has been adopted in the taxonomies of Nelson & al. (1983) and Burgess & al. (1988) for *F. oxysporum* and *F. solani* to provide utility for practical identification. However, these species are complex (Matuo & Snyder, 1973; Van Etten & Kistler, 1988) in a phylogenetic sense (Fig. 3). Thus far we have identified 5 phylogenetically distinct species within *F. oxysporum* (O'Donnell & Cigelnik, 1995b) and approximately 50 species within *F. solani* (O'Donnell & al., 1993; O'Donnell & Gray, 1995). In both species groups, morphological evolution has not kept pace with speciation (Larson, 1989). As a result, phylogenetically distinct homothallic and heterothallic species and all of Snyder & Hansen's (1953) form species are incorrectly viewed as strains of *F. solani* (Booth, 1971). Since many species within both of these polytypic species are of considerable importance as plant (Van Etten & Kistler, 1988) and animal pathogens (Nelson & al., 1994), there is a strong economic incentive to develop a natural classification and molecular tools for the accurate identification of these fungi. While Snyder & Hansen (1953) argued that morphologically cryptic taxa should not be formally described, preliminary estimates indicate that only about one-half of the phylogenetically distinct species within the *F. solani*-complex can be identified with morphology alone

(H. Nirenberg, pers. comm.). Viewing the problem from an evolutionary perspective, de Queiroz & Gauthier (1994) argue that taxonomists can only discover and name taxa because they exist in nature whether or not they are accurately reflected in a taxonomy. Clearly, a taxonomy that accurately reflects the phyletic diversity present within the *F. solani*-complex will require molecular tools for accurate identification of many isolates. For this reason, I anticipate that culture collections will need to provide a molecular-based identification service for *Fusarium* that would complement current identification services.

Under the International Code of Botanical Nomenclature, paraphyletic taxa (i.e., taxa in which some of the descendants of a common ancestor are excluded) such as *Fusarium*, *Nectria* and subgenus *Dialonectria* (Samuels & al., 1991) can be eliminated by either lumping or splitting in order to bring the taxonomy in line with the phylogenetic reconstruction (de Queiroz & Gauthier, 1994). While this will necessitate name changes, every attempt will be made to alter the current classification as little as possible (Wiley & al., 1991). Paraphyly of *Fusarium* and *Nectria* will be eliminated by recognizing less inclusive monophyletic groups. For example, our data indicate that *Fusarium* is paraphyletic with *Cylindrocarpon* derived within it and *Neocosmospora* and *Gibberella* are derived within *Nectria*. Rather than reduce *Cylindrocarpon* to synonymy with *Fusarium*, Fusaria basal to *Cylindrocarpon*, including subgenus *Dialonectria* and section *Coccophilum sensu* Booth (1971), will be recognized as a distinct anamorphic genus. Also, subgenus *Dialonectria* (Samuels & al., 1991) may be polyphyletic as it presently includes species with anamorphs other than *Fusarium*.

A number of monophyletic lineages have been identified within Fusaria connected to *Nectria* (K. O'Donnell, unpubl.) and these will be formally recognized as segregate teleomorphic genera, completely replacing *Nectria*, which should be restricted to the *Nectria cinnabarina*-group (Rossman, 1989). Unfortunately, *Neocosmospora* will replace *Nectria* as the teleomorph for the *Fusarium* Sect. *Martiella*, resulting in a name change for the well-known *Nectria haematococca*-complex (Van Etten & Kistler, 1988; O'Donnell & Gray, 1995). This name change can be rationalized in part because all of the phylogenetically distinct species within this complex species (Fig. 4) will require species epithets too.

Initially we tested the type of *Neocosmospora, N. vasinfecta*, as a potential outgroup for rooting purposes based on the findings of Spatafora & Blackwell (1994). Suprisingly, we discovered that *N. vasinfecta* is actually a microconidial *Fusarium* that has lost the ability to produce macroconidia and septate ascospores. Loss of these two key morphological features are autapomorphies that have

obscured the correct taxonomic placement of both the anamorph and teleomorph of this species. This finding serves to illustrates that morphological (Booth, 1984; Windels, 1989) and polythetic definitions (Gams & Nirenberg, 1989) of *Fusarium* are artificial because they have not taken into account that some Fusaria may have permanently lost the ability to produce macroconidia. For our own cladistic studies of the genus, we have adopted a stem-based phylogenetic definition (de Queiroz & Gauthier, 1994), which reads as follows: *Fusarium* consists of *F. ventricosum* and all related species sharing a more recent common ancestor with *F. ventricosum* than with *Cylindrocarpon*. Any species within this clade, therefore, would be identified as *Fusarium*, irrespective of the absence of key morphological features.

All species within the *Fusarium solani*-complex selected for further study have at least two paralogous beta tubulin genes (Schardl & al., 1994; Tsai & al., 1994). Gene sequences of clones representing the three subclades within this complex indicate the paralogous tubulins are highly divergent (May & al., 1987), thus making it possible to construct two orthologous gene trees from the duplicated genes (Doyle, 1992). Assuming the level of resolution within the tubulins is comparable to what we observed within the *Gibberella fujikuroi*-complex (O'Donnell & Cigelnik, 1995b), it should be possible to obtain a highly resolved species phylogeny for this large and economically important lineage. These studies will include an *a posteriori* morphological analysis directed at discovering taxonomically relevant morphological characters. When key phenotypic characters are mapped onto the independently derived gene trees, it should be possible to gain a better appreciation of which characters best reflect natural relationships and are taxonomically relevant and which ones are homoplaseous (Taylor & LoBuglio, 1995). By examining phenotypic character states in the contex of a highly resolved genotypic phylogeny, these studies should gain the necessary rigor required to inform stable taxonomic revision.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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