MYCOTAXON

Volume 109, pp. 443–460 *July–September 2009*

Ascription of poorly defined taxa to taxonomic entities using molecular phylogenies: a case study on *Nodulisporium* sp. producers of nodulisporic acid

G. Platas[1*], V. Gonzaléz[2], J. Collado[1],
J. Martín[1], C. Montes[1] & F. Peláez[1]

[*]*Gonzalo.platas@medinaandalucia.es*
[1] *Centro de Investigación Básica, Merck Sharp and Dohme de España S. A. Josefa Valcárcel 38, Madrid 28027, Spain*
[2] *Instituto Madrileño de Investigación y Desarrollo Rural Agrario y Alimentario (IMIDRA), Finca "El Encín"; Ctra. NII, Km 38,200 Alcalá de Henares, Madrid 28800, Spain*

Abstract — DNA sequences of the ITS1-5.8S-ITS2 region, β-tubulin, and α-actin genes were obtained from a set of *Nodulisporium* sp. isolates producing the potent anti-parasitic agent, nodulisporic acid, and other related strains. These sequences were used to confirm the molecular homogeneity of these NA-producers, to determine their most closely related taxa within genus *Hypoxylon*, and to explore the phylogenetic relationships between these strains and other hypoxyloid sequences from public DNA databases. The results from all the loci analyzed show that this group of strains constitutes a monophyletic taxon. This taxon would be included within a cluster containing endophytic, tropical xylariaceous isolates lacking defined teleomorph and taxonomic status, together with several *Hypoxylon* species such as *H. investiens*, *H. kanchanapisekii,* and *H. anthochroum*.

Key words — phylogeny, *Xylariales,* xylariaceous

Introduction

The comparison of DNA sequences is an objective method for establishing phylogenetic affinities among strains isolated in different laboratories all over the world. Despite the current initiatives claiming to select a gene as a universal bar-coding marker, in practice each laboratory has its own criteria for choosing a determined gene or group of genes to assess the genetic affinities among strains of interest. This approach is very useful in building phylogenies when no previous information is available. Where previous information exists, these new phylogenies could miss important information otherwise obtained from

comparison of other gene sequences from related cultures already stored in DNA databases.

The genus *Hypoxylon* serves as good example of this situation. During the last decade, a substantial number of works have been published describing a new *Hypoxylon* species or inferring phylogenetic relationships within *Hypoxylon* using the ITS1-5.8S-ITS2 region (e.g. Sánchez-Ballesteros et al. 2000, Lee et al. 2000, Mazzaglia et al. 2001, Guo et al. 2000, Promputtha et al. 2005, Suwannasai et al. 2005, Triebel et al. 2005, Peláez et al. 2008). On the other hand, Hsieh et al. (1995) published a comprehensive paper comparing sequences of two nuclear genes, β-tubulin and α-actin, from 109 strains and 3 specimens representing 83 taxa of *Xylariaceae*. However, only a few *Hypoxylon* species are represented by these three genes in GenBank, and usually the sequences are derived from different isolates (some of which may even have been misidentified), which might lead to wrong conclusions arising from incorrect information in new phylogenetic studies.

Our study revises the taxonomic position of a set of isolates representing a possibly undescribed *Nodulisporium* taxon producing nodulisporic acid (NA) (Polishook et al. 2001) by comparing the sequences of three different genomic regions — ITS, β-tubulin, and α-actin — with homologous sequences available in GenBank. Nodulisporic acid (Ondeyka et al. 1997) is an indole diterpene with very potent oral anti-flea activity in dogs that lacks overt mammalian toxicity (Ostlind et al. 1997, Shoop et al. 2001). Nodulisporic acid acts by activating a glutamate-gated chloride channel in insects (Smith et al. 2000). After the discovery of NA, six additional compounds from the same family were isolated from fermentation broths of the same producing strain (Ondeyka et al. 2002, Singh et al. 2004). Other derivatives have been chemically synthesized (Smith et al. 2006a,b, 2007a,b). NA biosynthesis research by Byrne et al. (2002) shows indole-3-glycerolphosphate as the precursor of the NA indole moiety, and Ireland et al. (2008) have recently reported the tryptophan synthetase gene related to NA production.

Nodulisporic acid was detected in extracts derived from 13 fungal strains isolated from different natural substrata from seven tropical locations in four continents. Detailed analyses of micromorphology and metabolic profiles, as well as of ITS sequences allowed their classification as members of a possible new biological species of the genus *Nodulisporium*, but no specific epithet has been assigned to this species. Although its teleomorph is still unknown, comparison of ITS sequences from other xylariaceous fungi suggested that it would most likely be a *Hypoxylon* species near *H. fendleri* Berk. ex Cooke (Polishook et al. 2001).

ITS region sequence data show isolates arranged into three groups according to ITS1 lengths of 210, 302, and 346 nucleotides respectively, due to a VNTR

(Variable Number of Tandem Repeats) polymorphism. Tandem repeats vary in number from two to five repetitions per isolate. Repeated units are rather variable and encompass three tandem repeated motifs of 11 nucleotides. The different size of this subunit could be explained by slipped-strand mispairing occurrences (Platas et al. 2002). In contrast, ITS2 sequences were much more homologous. These data suggest that all isolates could be considered as representing the same taxon, although divided into populations showing some degree of genetic differentiation.

Materials and methods

Fungal strains

Of 13 *Nodulisporium* sp. strains producing NA (Polishook et al. 2001) investigated, 3 were excluded as representing duplicates and 10 were selected for this analysis. All these strains are coded with their accession numbers in the Merck Culture Collection (MF); those included in this study accompanied by their geographical origin, ITS1 gene size, and GenBank sequence accession numbers are listed in Table 1.

DNA extraction, PCR amplification and sequencing

DNA was extracted according to Peláez et al. (1996). The ITS1-5.8S-ITS2 region was amplified using universal primers ITS4 and ITS5 (White et al. 1990). β-tubulin and a fragment of α-actin genes were obtained using PCR primers T1/T22 (O'Donnell & Cigelnik 1997) and ACT-512F/ACT-783R (Carbone & Kohn 1999) respectively. PCR amplification followed standard procedures (40 cycles of 30 s at 93°C, 30 s at 53°C, 2 min at 72°C). About 0.1 μg/ml of the double stranded amplification products were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Norwalk CT) following manufacturer recommended procedures. Purified PCR products were directly sequenced using the same primer pairs as in the PCR reactions. Inner primers were used to complete the sequence of β-tubulin gene: T21, T10, T11, T12 and T224 (O'Donnell & Cigelnik 1997). An additional primer bt_1 5′-CTTATGTTTACTGCTGACCC-3' derived from the 5' end of an initial β-tubulin sequence alignment was developed to amplify DNA of species that failed in the initial attempt. Partial sequences obtained in sequencing reactions were assembled with Genestudio 2.1.1.5. (Genestudio, Inc., Suwanee, GA, USA). DNA sequences were aligned with Genestudio 2.1.1.5. Multiple alignments were deposited in TreeBASE with number SN4083.

Culture description

Culture descriptions and morphology were observed directly using an optical microscope (Leica DML52) with an image capture device. Microscope slides of the fungal cultures (including either somatic mycelia or conidiogenic structures) were prepared under a binocular lens Leica Wild M3C with cold light device and mounted in distilled water or lactophenol-cotton blue as needed. Cultures were identified by direct observation and measurements of the vegetative and reproductive structures.

Table 1. Isolates studied.

Species	Strain code	Substrate	Country	ITS1 size	GenBank accession numbers		
					ITS1-5.8S-ITS2	β-tubulin	α -actin
Annulohypoxylon multiforme	F160843		USA	193	FJ185303	FJ185282	-
Annulohypoxylon cohaerens	F160842		USA	190	FJ185301	FJ185283	FJ185264
Annulohypoxylon cohaerens	F119894	*Quercus faginea*	Spain	190	FJ185302	FJ185286	FJ185267
Daldinia concentrica	F090108		Papua New Guinea	177	FJ185300	FJ185285	FJ185280
Nodulisporium sp.	MF6245	Lichen	Puerto Rico	210	AF201756	FJ185296	FJ185272
Nodulisporium sp.	MF6263	Bush twigs	Colombia	346	AF201749	FJ185292	FJ185273
Nodulisporium sp.	MF6324	Dead leaves	Peru	210	AF201759	FJ185289	FJ185276
Nodulisporium sp.	MF6321	Leaf litter	Mauritius Island	346	AF201751	FJ185297	FJ185275
Nodulisporium sp.	MF6377	*Scaevola plumieri*	Eq. Guinea	210	AF201757	FJ185287	FJ185277
Nodulisporium sp.	MF6378	*Anacardium occidentale*	Eq. Guinea	210	AF201760	FJ185295	FJ185278
Nodulisporium sp.	MF6379	*Dorstenia elliptica*	Eq. Guinea	210	AF201761	FJ185290	FJ185279
Nodulisporium sp.	MF5954 ATCC 74245	Stems of *Bontia daphnoides*	Hawaiian Islands	302	AF201753	FJ185291	FJ185270
Nodulisporium sp.	MF6230	Horse dung	Marquesas Islands	302	AF201752	FJ185288	FJ185271
Nodulisporium sp.	MF6315	Leaf litter from coffee plant	Mauritius Island	302	AF201754	FJ185293	FJ185274
Nodulisporium sp.	F089878	Jungle pond	French Guyana	210	FJ185305	FJ185294	FJ185268
Hypoxyloid isolate	JP5613 CBS 123520	Oregano leaves	Mexico	184	FJ185306	FJ185284	FJ185269
Hypoxylon subrutiloides	F202416	Fallen wood	New Zealand	210	FJ185304	FJ185281	FJ185263
Hypoxylon investiens	CBS 118183		Malaysia	254	FJ185307	FJ185298	FJ185265
Hypoxylon investiens	CBS 118185		Ecuador	258	FJ185308	FJ185299	FJ185266

Phylogenetic analysis

Bayesian inference and Markov chain Monte Carlo simulations (MCMC) were used to generate phylogenetic hypotheses from individual gene data sets using MrBayes 3.01 (Huelsenbeck et al. 2002, Ronquist & Huelsenbeck 2003). To improve mixing of the chain, four incrementally heated simultaneous MCMCs were run over 2,000,000 generations. MrModeltest 2.2 (Nylander 2004) was used to perform hierarchical likelihood ratio tests to calculate the Akaike information criterion (AIC) values of the nucleotide substitution models. The HKY+I+G model was selected by AIC for aligning the β-tubulin and α-actin gene fragment that allowed two classes of substitution types, a portion of invariant alignment positions, and mean substitution rates varying across the remaining positions according to a gamma distribution. Due to the high ITS1 variability in the *Xylariales* found in previous studies (Platas et al. 2000), the ITS1 section containing tandem repeats was removed from analysis. The HKY+G model was selected by AIC for aligning the ITS1-5.8S-ITS2 fragment, which allowed two classes of substitution types and mean substitution rates varying across the remaining positions according to a gamma distribution. Priors used for the MCMC process were a Dirichlet distribution for substitution rates and nucleotide frequencies, and a uniform prior for the rate parameter of the gamma distribution. For all these analyses, the sampling frequency at which the trees were stored was 100, the 1000 first trees were discarded, and a majority rule consensus tree was obtained.

Induction of ascoma production

A method was introduced to artificially induce production of the teleomorph state of the NA-producing *Nodulisporium* sp. and the hypoxyloid strain JP5613. Control strains of *Hypoxylon investiens* (Schwein.) M.A. Curtis were tested in the same conditions. The fungal strains were inoculated into 3–6-year old sterilized twigs (3–5 cm diam × 8–10 cm long) taken from healthy *Citrus aurantium* L. and *C. reshni* Hort. ex Tanaka. As members of the *Xylariales* are believed to be heterothallic, several fungal strains were inoculated into the same wood pieces to induce ascoma production. Fungal inoculum of millet grain colonized by the fungi in solid-state fermentation conditions was subsequently dehydrated and inserted into holes drilled in the wood. Inoculated branches were then incubated in sterilized flasks at room temperature for 120 days.

Results

Comparison of ITS-5.8S-ITS region with in-house DNA sequence databases

As the ITS1 sequence of the NA-producers was shown to be very variable (Platas et al. 2002), the NA-producer ITS2 sequences, which showed 94–100% homology among the isolates, was used to find similar sequences within our proprietary DNA database using BLAST analysis. The list of best matches and the percentage of similarity includes: *Nodulisporium* sp. F089878 (96–100%), xylariaceous sp. JP5613 (88–91%), *Annulohypoxylon cohaerens* (Pers.) Y.M. Ju et al. F119894 & F160842 (79–81 %), *Annulohypoxylon multiforme* (Fr.) Y.M. Ju et al. F160843 (78–80%), *Daldinia concentrica* (Bolton) Ces. & De Not. F090108 (75–77% homology), and *Hypoxylon subrutiloides* Y.M. Ju & J.D.

Rogers F202416 (75–77%). All these strains were selected for the amplification of β-tubulin and α-actin genes.

PCR amplification of the ITS, α-actin and β-tubulin genes

Amplification of the ITS region and α-actin gene fragment was successful for all except *Annulohypoxylon multiforme* F160843, whose α-actin gene could not be amplified. Initially, β-tubulin gene amplification was not successful in *Nodulisporium* sp. MF6245, MF6263, and MF6321, *Annulohypoxylon multiforme* F160843, and *Daldinia grandis* Child F090108 using the primer combination T1/T22. A new primer (bt_1) was designed based upon the sequences from the 5' end of the gene, which combined with primer T22 successfully amplified the remaining strains.

Sequence comparisons and BLAST analyses

The similarity percentage range between the NA-producing *Nodulisporium* sp. for β-tubulin (1466 bp) and α-actin (268 bp) gene sequences was 94–100%. The similarity range between *Nodulisporium* sp. strain F089878 and the NA-producers was within the same interval (94–100%) for both genes. This data, together with the morphological observations (see below), confirmed initial conclusions based on the ITS2 sequence, suggesting that isolate F089878 would be co-specific with the NA-producers. Strain JP5613 showed 87–88% similarity for β-tubulin and 79–80% for α-actin gene. Similarities found among the remaining sequenced isolates were not significant.

A BLAST analysis of α-actin and β-tubulin NA-producer gene sequences was performed against GenBank. The best matching sequences were aligned, and preliminary phylograms were obtained using MCMC analysis. In the phylograms obtained using both genes (data not shown), the clade of NA-producing *Nodulisporium* sp. strains clustered together with *Hypoxylon investiens* BCRC34074 from Taiwan (87–90% similarity for α-actin and 87–88% for β-tubulin respectively). For confirmation, two new *H. investiens* strains from Malaysia (CBS 118183) and Ecuador (CBS 118185) were included and their ITS region, β-tubulin, and α-actin gene fragments were sequenced. These two strains showed a homology with the published *H. investiens* sequences of 92–95% (β-tubulin) and 94–96% (α-actin). The similarity percentage between these two isolates for the whole ITS1-5.8S-ITS2 region was 90%, confirming the high variability previously found in the ITS of conspecific *Xylariales* (Sánchez-Ballesteros et al. 2000). The similarity range between the ITS2 of those strains with the NA-producers was 84–92%.

BLAST analysis of the NA-producer ITS1-5.8S-ITS2 regions against GenBank revealed significant matches with sequences of some endophytic strains or herbarium specimens, mainly from tropical Asia. The best matches included

GenBank AF153746 and AF153742, two different morphospecies isolated from *Livistona chinensis* (Jacq.) R. Br. now assigned to the *Xylariales* (Guo et al. 2000). Another related ITS sequence (DQ485958) belongs to a xylariaceous endophyte from *Magnolia liliifera* Baill. tentatively classified within *Hypoxylon* near *H. fendleri* by Promputtha et al. (2005). All these sequences correspond to strains lacking any known teleomorph. Together with these xylariaceous endophytes, BLAST analysis found also matches with several *Hypoxylon* spp.: *H. rubiginosum* (Pers.) Fr. (DQ233758, DQ233759), *H. anthochroum* Berk. & Broome (DQ201125, DQ201126), and *H. kanchanapisekii* Suwann. et al. (DQ233741, DQ233742, DQ233743). Unfortunately, none of these strains were available for further studies.

Phylogenetic analyses

Bayesian analyses of β-tubulin and α-actin sequence alignments were performed including sequences obtained from: i) 11 NA-producing *Nodulisporium* sp isolates; ii) *Nodulisporium* sp. F089878; iii) xylariaceous strain JP5613; iv) *H. investiens*, the presumed closest relative to the NA-producers; v) *Annulohypoxylon cohaerens* F119894 and F160842, *A. multiforme* F160843, *Daldinia concentrica* F090108, and *Hypoxylon subrutiloides* F202416, whose ITS2 genes were retrieved as best matches with the NA-producing strain sequences from our in-house DNA database; and vi) three groups of sequences representing miscellaneous taxa from *Hypoxylon* s. str., *Annulohypoxylon,* and *Daldinia* that were the best matches from the GenBank BLAST search. In addition, a third phylogenetic ITS sequence analysis was performed for Strains (i)–(v) above and sequences from assorted xylariaceous fungi ranked as best BLAST search matches with the NA-producing strains.

α-actin and β-tubulin phylogenies

In general, the arrangement of the NA-producing strains in the phylogenetic trees derived from the α-actin (Fig. 1) and β-tubulin (Fig. 2) gene fragments was consistent with previous ITS sequence studies (Polishook et al. 2001). All formed a natural monophyletic group, suggesting their ascription to a single biological species.

Both phylogenetic trees placed *H. investiens* as the closest relative of the NA-producing strains, together with xylariaceous isolate JP5613. All sequences fell into a clade with high credibility support in both trees. However, evolutionary relationships of these isolates with the other hypoxyloid genera remain unclear. Thus, the α-actin based phylogeny suggests (without clade credibility support) a relationship between NA-producers and one node containing *Hypoxylon* s. str. taxa, whereas the the β-tubulin phylogenetic reconstruction topology shows a clade containing *Annulohypoxylon* species as the group most closely related to the NA-producers, supported with 98% clade credibility.

Both phylogenetic trees are consistent with the well-known heterogeneity of *Hypoxylon* s. lat. (Sánchez-Ballesteros et al. 2000, Hsieh et al. 2005, Peláez et al. 2008). In both trees, *Daldinia* and *Annulohypoxylon* spp. sequences group in two well supported clades, confirming data from Hsieh et al. (2005), but taxa representing the modern concept of *Hypoxylon* s. str. occur on several separate branches, suggesting a polyphyletic origin.

Distribution of NA-producing strains within their clade differs in both phylogenetic trees. In both trees, isolates with a medium to large ITS1 intermingle and cluster consistently as a monophyletic group with total bootstrap support, but the distribution of isolates with a small ITS1 differs. They form a monophyletic group without statistical support in the α-actin based phylogeny but split into two groups in the β-tubulin based tree.

With few exceptions, distribution of the NA-producing strains in the trees does not correlate with geographic origin. For instance, two (MF6377, MF6378) of the three sequences from Equatorial Guinea group together in both trees with the third isolate (MF6379) more distant. Within the group of short ITS1 sequence isolates, isolate F089878 clusters with strain MF6245, which could reflect their close geographic origins (French Guyana and Puerto Rico, respectively). Interestingly, in both phylogenies, the two isolates from Mauritius Island (MF6315, MF6321) cluster together within a well supported clade despite their different ITS1 sizes.

ITS phylogeny

In the phylogram generated from the ITS sequence analyses (Fig. 3), the NA-producing strains form a monophyletic group with high credibility support, as expected. The NA-producers are distributed according to their ITS1 size in two well supported monophyletic clades, with the small ITS1 strains separate from medium–large ITS1 strains, as already reported (Polishook et al. 2001). This clade also includes *Nodulisporium* sp. strain F089878 as well as a sequence from an unidentified xylariaceous fungus (AF153746) isolated from *Livistona chinensis* (Guo et al. 2000).

The clade containing the NA-producing strains falls within a larger branch with high (97%) clade credibility support that also includes sequences from several *Hypoxylon* species (e.g., *H. kanchanapisekii*, *H. anthochroum*, *H investiens*, *H. rubiginosum*) and several undetermined xylariaceous fungi. Two undetermined xylariaceous (AF153742, DQ485958) sequences cluster with *H. investiens* sequences in a well-supported clade. Interestingly, *Hypoxylon fendleri*, the closest reported match to the NA-producing strains (Polishook et al. 2001), occurs outside this clade.

A sister clade to this large monophyletic group, supported with 100% of clade credibility, comprised sequences from *H. rubiginosum*, *H. petriniae* M.

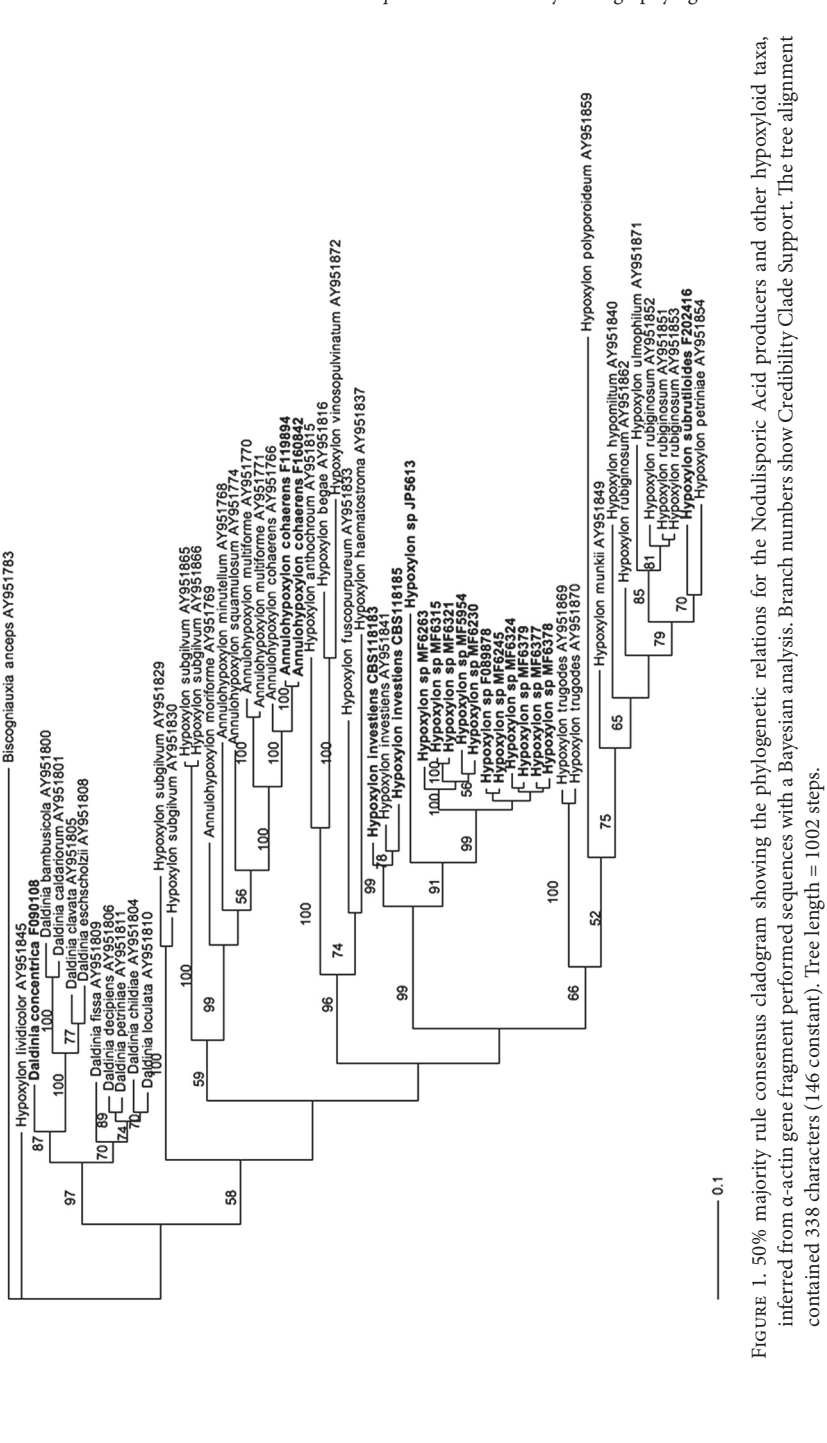

FIGURE 1. 50% majority rule consensus cladogram showing the phylogenetic relations for the Nodulisporic Acid producers and other hypoxyloid taxa, inferred from α-actin gene fragment performed sequences with a Bayesian analysis. Branch numbers show Credibility Clade Support. The tree alignment contained 338 characters (146 constant). Tree length = 1002 steps.

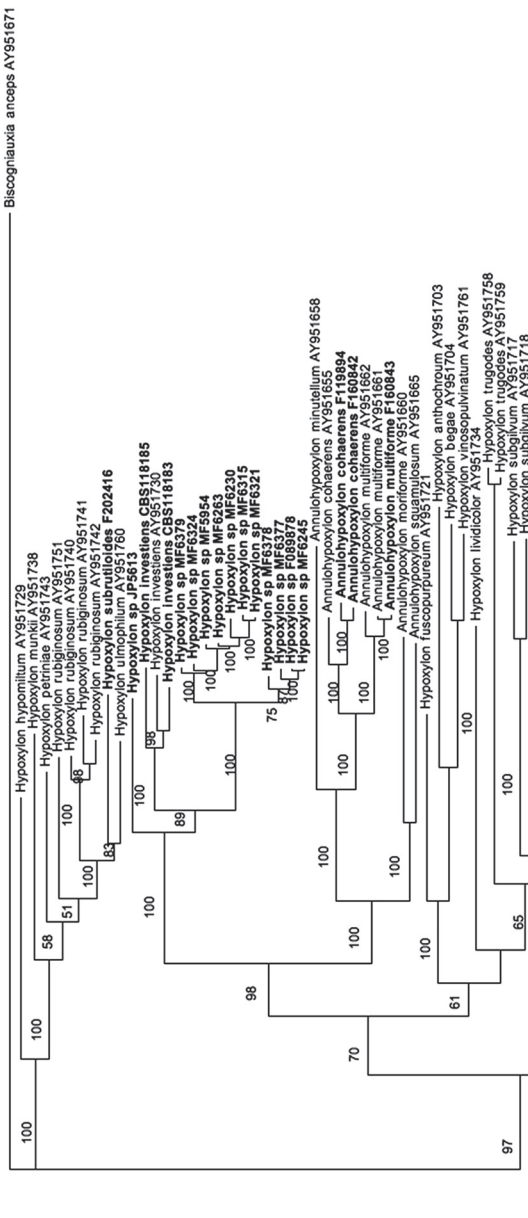

FIGURE 2. 50% majority rule consensus cladogram showing the phylogenetic relations for the Nodulisporic Acid producers and other hypoxyloid taxa, inferred from β-tubulin gene sequences performed with a Bayesian analysis. Branch numbers show Credibility Clade Support. The tree alignment contained 1659 characters (808 constant). Tree length = 4762 steps.

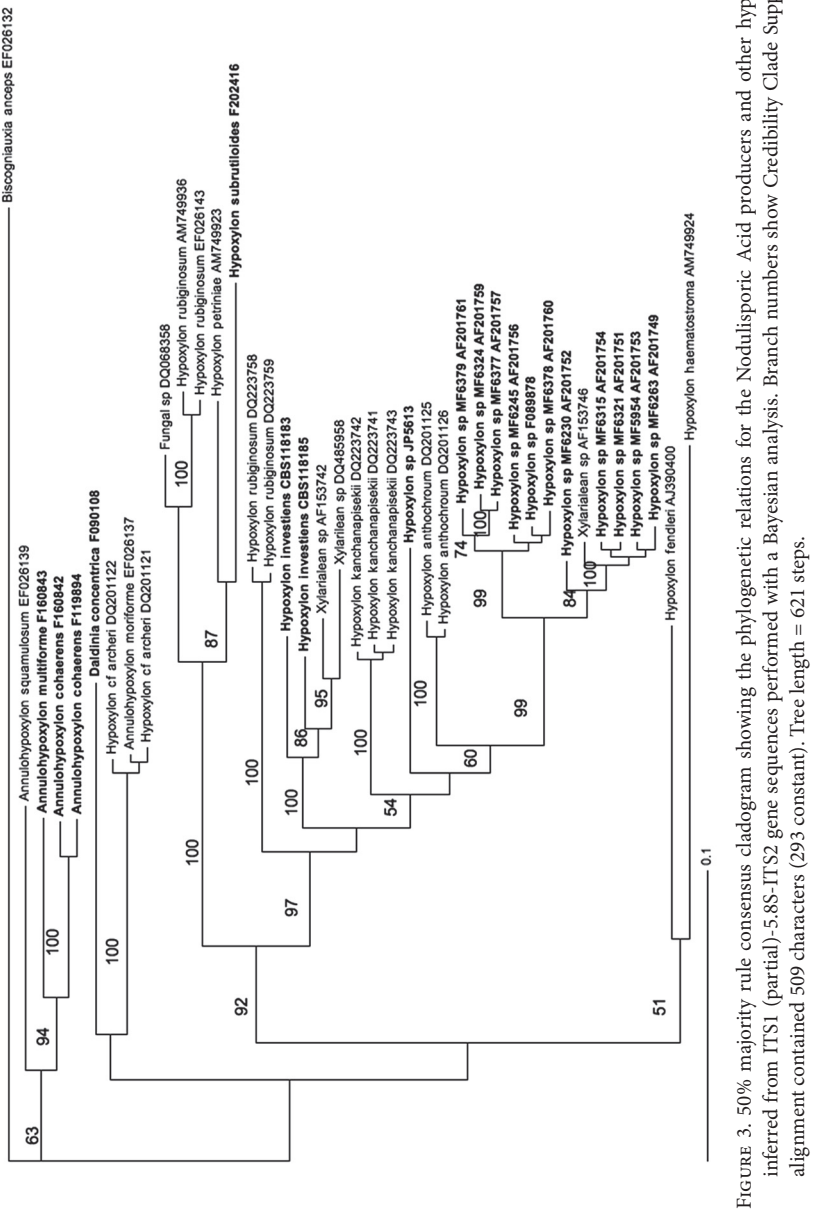

FIGURE 3. 50% majority rule consensus cladogram showing the phylogenetic relations for the Nodulisporic Acid producers and other hypoxyloid taxa, inferred from ITS1 (partial)-5.8S-ITS2 gene sequences performed with a Bayesian analysis. Branch numbers show Credibility Clade Support. The tree alignment contained 509 characters (293 constant). Tree length = 621 steps.

Stadler & J. Fourn. and *H. subrutiloides*, together with an undetermined fungal species (DQ068358).

Morphological features

Despite our attempts to induce teleomorph production by growing the NA-producing strains on wood twigs, no stromata or perithecia developed under the tested conditions. Thus, plated colonies were examined for morphological features common to the phylogenetically related taxa. As previously reported (Polishook et al. 2001), cultural characters of the NA-producing strains were remarkably homogeneous. All strains shared the same optimal growth temperature (37ºC), the distinctive (i.e., "*Nodulisporium*-like") and characteristically sweet aromatic odour, and a *Nodulisporium* anamorph composed of mononematous, erect conidiophores that are penicillately branched (verticillate), covered in patches by a brown melanin-like parietal pigment and with conidiogenous minutely verruculose and cylindrical terminal and holoblastic conidiogenous cells bearing oblong-elliptical, smooth, hyaline conidia that fail to grow after germination, are produced sympodially from the conidiogenous cell apices, and accumulate in apical clusters. Another common feature was the abundant brownish-black soluble pigment produced in both plate and liquid culture.

Isolate F089878, a xylariaceous endophyte from French Guyana, produced colonies indistinguishable from the NA-producing strains with respect to *Nodulisporium* anamorph morphology, optimal growth temperature, and pigment production. These findings — consistent with the results derived from DNA sequence analyses— suggest that isolate F089878 is conspecific with the NA-producers, although nodulisporic acid production has not yet been verified.

Hypoxyloid fungus JP5613 (isolated from *Origanum* leaves from Mexico) differed macroscopically. This fungus produced whitish to pale grey-white colonies lacking the characteristic sweet "*Nodulisporium*-like" odour. Furthermore, the *Nodulisporium* anamorph was not observed in any of the cultures, where only intercalary and/or terminal chains of globose, thick-walled chlamydospores were seen. Although not very common in the genus, these resistance structures have been reported for some *Hypoxylon* species, such as *H. ticinense* L.E. Petrini, *H. subticinense* Y.M. Ju & J.D. Rogers (Ju & Rogers 1996) and *H. haematostroma* Mont. (Rogers et al. 1987).

Together with these isolates, two *Hypoxylon investiens* strains (CBS 118183, CBS 118185) were also examined in plate culture. These strains exhibited greenish olivaceous tones when young, becoming hazel with creamy-white margins at the maturity, different than the light orange yellow to pale reddish tones recorded for NA-producer strain cultures. Moreover, *H. investiens*

produces in culture a fertile, *Periconiella*-like anamorph (Ju & Rogers 1996) different from the *Nodulisporium* anamorph observed in the NA-producers.

Concerning *H. kanchanapisekii*, no descriptions of anamorph morphology were found in the literature and no strains were available allowing comparison with the NA-producers.

Discussion

Phylogenetic species recognition has become a powerful inference tool to detect taxa that are cryptic or poorly defined based on traditional diagnostic features (Taylor et al. 2000). There are numerous examples of fungal groups where analyses of multiple gene sequence data have allowed recognition of existing genetic diversity in genera like *Aspergillus* (Geiser et al. 2007), *Lasiosphaeria* (Miller & Huhndorf 2004), and *Neurospora* (Dettman et al. 2003) or species complexes such as *Neurospora discreta* D.D. Perkins & N.B. Raju (Dettman et al. 2006), and *Serpula himantioides* (Fr.) P. Karst. (Kauserud et al. 2005).

As stated above, the NA-producing strains included in this study shared a similar culture phenotype despite their different substrates and geographical origins. In addition, those strains consistently formed a monophyletic taxon based on phylogenetic analyses of three sets of sequences (ITS region, β-tubulin gene, α-actin partial gene). The relative position of the NA-producers in the phylograms supports the existence of a biological or phylogenetic species clearly differentiated from other related taxa by nucleotide divergence rates in several independent loci.

The NA-producers have an unusually variable ITS1 size caused by VNTR duplications. Duplication of this VNTR may have taken place independently in different populations. Isolates MF6321 (ITS1 size 346 bp) and MF6315 (302 bp), both recovered from samples collected in Mauritius Island, clustered together in the β-tubulin and α-actin gene phylograms (Figs. 1–2). Moreover, they have identical mutations in the second and third tandem repetitions. Thus, our data suggest that MF6321 is more closely related to MF6315 than to the only other large ITS1 isolate (MF6263 from Colombia).

Xylariaceous strain MS1095 (sequence AF153746) appears most likely conspecific with the NA-producing strains. Its medium-sized ITS1 sequence (292 bp) clusters in the NA-producers clade with the medium- to large-sized ITS1 strain sequences (Fig. 3). Unfortunately this strain is no longer available for study, so we could not check for NA production.

This ITS1 size variation is not unique to NA-producers. The ITS1 sequences of *Hypoxylon investiens* and *Hypoxylon* sp. DQ485958 also show an unusual length. Strain CBS 118183 has 9 tandem repeated motifs plus one degenerate repetition, and CBS 118185 and *Hypoxylon* sp. DQ485958 sequences have 10 tandem repeated motifs.

In contrast with previous studies (Polishook et al. 2001), which suggest *H. fendleri* as the most closely related taxon, our newly obtained molecular sequence data indicate a close relationship between NA-producers and other tropical *Hypoxylon* and xylariaceous endophytes. *Hypoxylon investiens, H. kanchanapisekii*, *H. anthochroum* DQ223758-9 and *H. rubiginosum* DQ201125-6 appear to be the most closely related to the NA-producing xylariaceous fungi. Compared to the NA-producing species, *Hypoxylon investiens* develops a *Periconiella*-like anamorph in culture and has a different geographical distribution, including temperate zones of the Northern Hemisphere. *Hypoxylon kanchanapisekii,* with no reported anamorphic state, is a recently described taxon inhabiting bamboo stems from Thailand that is morphologically close to *H. parksianum* Y.M. Ju & J.D. Rogers (Suwannasai et al. 2005). *Hypoxylon investiens* and *H. parksianum* also share some features that place them close in morphological taxonomic keys (Ju & Rogers 1999). *Hypoxylon anthochroum* and *H. rubiginosum* have been considered as synonymous by some authors (e.g. Miller 1961), although results from other studies (e.g. Ju & Rogers 1996, Hsieh et al. 2005) do not support that synonymy. Our α-actin and β-tubulin phylograms and other consistent results (Hsie et al. 2005) show *H. anthochroum* as quite distant from *H. investiens*, while the ITS phylogeny seem to suggest a closer relationship.

The ITS phylogeny clusters the *H. rubiginosum* sequences on two separate branches (two each, respectively). The *H. rubiginosum* sequences included in the *H. petriniae-H. subrutiloides* cluster originated from two different studies, and their position in the trees is consistent with previous data derived from α-actin and β-tubulin sequence data (Hsie et al. 2005). However, two different *H. rubiginosum* strains belong to a clearly separate clade. This could either be due to misidentification of some of the sequence data or reflect the well-known heterogeneity of this taxon, usually considered as a large species complex, where numerous varieties have been split into different new taxa (Petrini & Müller 1986). This would be the case for *H. rubiginosum* var. *cercidicola* (Berk. & M.A. Curtis) L.E. Petrini (Petrini & Müller 1986) and *H. cercidicola* (Berk. & M.A. Curtis) Y.M. Ju & J.D. Rogers (Granmo 1999), two homotypic synonyms representing *Diatrype cercidicola,* which now accommodates isolates with thin, vinaceous stromata and *Virgariella*-like anamorphs that have a secondary metabolite profile clearly different from *H. rubiginosum* (Stadler et al. 2004). The separation of *H. petriniae* from *H. rubiginosum* is supported by the results shown in Figs. 1–3.

According to our data above, the NA-producing species must be assigned to genus *Hypoxylon* s. str. Previous studies have suggested that the NA-producers belong to a unique and complex biological species. Thus, Triebel et al. (2005), using 5.8S-ITS phylogenies, observed that the NA-producers constitute a defined

clade, separate from other hypoxyloid taxa, both containing specific ITS1 and ITS2 sequence. In addition, unpublished results cited in Stadler & Hellwig (2005) indicate that these isolates produce neither naphthalens nor melleins, metabolites usually found in the two main lineages in the *Hypoxyloideae*. To our knowledge, *H. investiens* and *H. kanchanapisekii* have not been tested for the production of those compounds. It would be interesting to know whether these species, which appear more or less related to NA-producers based on sequence analyses, also share this chemotaxonomic feature. If so, this could provide an additional evidence for segregation of the NA-producers and related taxa from *Hypoxylon* s. str.

In summary, this work presents evidence for the phylogenetic recognition of an endophytic, hypoxyloid, pantropical undescribed species that produces nodulisporic acid. This is another example of the usefulness of molecular methods to detect and define new taxonomical entities, especially in those cases where diagnostic data from both teleomorphic and anamorphic states are scarce or even unavailable, and where other evidence (e.g., metabolic profiles, cultural features) suggests the existence of an undescribed taxon.

Acknowledgments

The authors wish to thank Jon Polishook for providing the nodulisporic acid producing strains, Ana Pérez for her technical support, and Drs. Victor Rubio and Alberto Miguel Stchigel for pre-submission review. We wish to dedicate the present work to all the people whose personal effort helped to create and keep in business the Centro de Investigación Básica de España (CIBE) during 53 productive years of microbial natural products research, until Merck & Co. closed CIBE in September 2008 due to an unfortunate "strategic decision."

Literature cited

Alfaro ME, Zoller S. Lutzoni F. 2003. Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. Molecular Biology and Evolution 20: 255–266.

Byrne KM, Smith SK, Ondeyka JG. 2002. Biosynthesis of nodulisporic acid A: precursor studies. Journal of the American Chemical Society 124: 7055–7076.

Dettman JR, Jacobson DJ, Turner E, Pringle A, Taylor JW. 2003. Reproductive isolation and phylogenetic divergence in *Neurospora*: Comparing methods of species recognition in a model eukaryote. Evolution 57: 2721–2741.

Dettman JR, Jacobson DJ, Taylor JW. 2006. Multilocus sequence data reveal extensive phylogenetic species diversity within the *Neurospora discreta* complex. Mycologia 98: 436–446.

Carbone I, Kohn LM. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91: 553–556.

Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, Samson RA. 2007. The current status of species recognition and identification in *Aspergillus*. Studies in Mycology 59: 1–10.

Granmo A. 1999. Morphotaxonomy and chorology of the genus *Hypoxylon* (*Xylariaceae*) in Norway. Sommerfeltia 26: 1–81.

Guo LD, Hyde KD, Liew CY. 2000. Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. New Phytologist 147: 617–630.

Huelsenbeck JP, Larget B, Miller RE, Ronquist F. 2002. Potential applications and pitfalls of Bayesian inference of phylogeny. Systematic Biology 51: 673–688.

Hsieh HM, Ju YM, Rogers JD. 2005. Molecular phylogeny of *Hypoxylon* and closely related genera. Mycologia 97: 844–865.

Ireland C, Peekhaus N, Lu P, Sangari R, Zhang A, Masurekar P, An Z. 2008. The tryptophan synthetase gene TRP1 of *Nodulisporium* sp.: molecular characterization and its relation to nodulisporic acid A production. Applied Microbiology and Biotechnology 79: 451–459.

Ju YM, Rogers JD. 1996. A revision of the genus *Hypoxylon.* Mycologia Memoir No. 20. St. Paul: APS Press. 365 p.

Ju YM, Rogers JD. 1999. The *Xylariaceae* of Taiwan (excluding *Anthosthomella*). Mycotaxon 73: 343–440.

Kauserud H, Stensrud O, Decock C, Shalchian-Tabrizi K, Schumacher T. 2005. Multiple gene genealogies and AFLPs suggest cryptic speciation and long-distance dispersal in the basidiomycete *Serpula himantioides* (*Boletales*). Molecular Ecology 15: 421–431.

Lee JS, Ko KS, Jung HS. 2000. Phylogenetic analysis of *Xylaria* based on nuclear ribosomal ITS1-5.8S-ITS2 sequences. FEMS Microbiological Letters 187: 89–93.

Mazzaglia A, Anselmi N, Vicario S, Vannini A. 2001. Sequence analysis of the 5.8S rDNA and ITS regions in evaluating genetic relationships among some species of *Hypoxylon* and related genera. Mycological Research 105: 670–675.

Miller AN, Huhndorf SM. 2004. Using phylogenetic species recognition to delimit species boundaries within *Lasiosphaeria*. Mycologia 96: 1106–1127.

Miller JH. 1961. A monograph of the world species of *Hypoxylon*. Univ. Georgia Press. Athens. 158 pp.

Nylander JA. 2004. MrModeltest v2. Evolutionary Biology Centre, Uppsala University.

O'Donnell K, Cigelnik E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Molecular Phylogenetics and Evolution 7: 103–116.

Ondeyka JG, Helms GL, Hensen OD, Goetz MA, Zink DL, Tsipouras A, Shoop WL, Slayton L, Dombrowski AW, Polishook JD, Ostlind DA, Tsou NN, Ball RG, Singh SB. 1997. Nodulisporic Acid A, a novel and potent insecticide from a *Nodulisporium* sp. isolation, structure determination and chemical transformations. Journal of the American Chemical Society 119: 8809–8816.

Ondeyka JG, Dahl-Roshak AM, Tkacz JS, Zink DL, Zakson-Aiken M, Shoop WL, Goetz MA, Singh SB. 2002. Nodulisporic Acid B, B1, and B2: A Series of 10-Deoxy-nodulisporic Acids from *Nodulisporium* sp. Bioorganic and Medicinal Chemistry Letters 12: 2941–2944.

Ostlind DA, Felcetto T, Misura A, Ondeyka JG, Smith S, Goetz MA, Shoop WL, Mickle W. 1997. Discovery of a novel indole diterpene using first instars of *Lucilia sericata*. Medical and Veterinary Entomology 11: 407–408.

Peláez F, Platas G, Collado J, Díez MT. 1996. Infraspecific variation in two species of aquatic hyphomycetes, assessed by RAPD analysis. Mycological Research 100: 831–837.

Peláez F, González V, Platas G, Sánchez-Ballesteros J, Rubio V. 2008. Molecular phylogenetic studies within the family *Xylariaceae* based on ribosomal DNA sequences. Fungal Diversity 31: 111–134.

Petrini LE, Müller E. 1986. Haupt-und Nebenfruchtformen europäischer Hypoxylon-Arten (*Xylariaceae, Sphaeriales*) und verwandter Pilze. Mycologia Helv. 1(7): 501–627.

Platas G, Acero J, Borkowski JA, González V, Portal MA, Rubio V, Sánchez-Ballesteros J, Salazar O, Peláez F. 2001. Presence of a simple tandem repeat in the ITS1 region of the *Xylariales*. Current Microbiology 43: 43–50.

Platas G, Polishook JD, Peláez F. 2002. VNTR polymorphism in the ITS1 region (rDNA) in isolates of a *Nodulisporium* species. Mycological Research 106: 1293–1298.

Polishook JD, Ondeyka JG, Dombrowski AW, Peláez F, Platas G, Teran AM. 2001. Biogeography and relatedness of *Nodulisporium* strains producing nodulisporic acid. Mycologia 93: 1125–1137.

Promputtha I, Jeewon R, Lumyong S, McKenzie EHC, Hyde KD. 2005. Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from *Magnolia liliifera* (*Magnoliaceae*). Fungal Diversity 20: 167–186.

Rogers JD, Callan BE, Samuels GJ. 1987. *Xylariaceae* of the rain forests of North Sulawesi (Indonesia). Mycotaxon 29: 113–172.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Sánchez-Ballesteros J, González V, Salazar O, Acero J, Portal MA, Julián M, Rubio V, Bills GF, Polishook JD, Platas G, Mochales S, Peláez F. 2000. Phylogenetic study of *Hypoxylon* and related genera based on ribosomal ITS sequences. Mycologia 92: 964–977.

Shoop WL, Gregory LM, Zakson-Aiken M, Michael BF, Haines HW, Ondeyka JG, Meinke PT, Schmatz DM. 2001. Systemic efficacy of nodulisporic acid against fleas on dogs. Journal of Parasitology 87: 419–423.

Singh SB, Ondeyka JG, Jayasuriya H, Zink DL, Ha SN, Dahl-Roshak A, Greene J, Kim JA, Smith MM, Shoop W, Tkacz JS. 2004. Nodulisporic acids D-F: structure, biological activities, and biogenetic relationships. Journal of Natural Products 67: 1496–1506.

Smith AB, Davulcu AH, Cho YS, Ohmoto K, Kürti L, Ishiyama H. 2007. Indole Diterpene Synthetic Studies. Total Synthesis of (+)-Nodulisporic Acid F and Construction of the Heptacyclic Cores of (+)-Nodulisporic Acids A and B and (–)-Nodulisporic Acid D. Journal of Organic Chemistry 72: 4596–4610.

Smith AB, Kürti L, Davulcu AH, Cho YS, Ohmoto K. 2007. Indole Diterpene Synthetic Studies: Development of a Second-Generation Synthetic Strategy for (+)-Nodulisporic Acids A and B. Journal of Organic Chemistry 72: 4611–4620.

Smith AB, Davulcu AH, Kürti L. 2006. Indole diterpenoid synthetic studies. The total synthesis of (+)-nodulisporic acid F. Organic Letters 8: 1665–1668.

Smith AB, Davulcu AH, Kürti L. 2006. Indole diterpenoid synthetic studies. Construction of the heptacyclic core of (–)-nodulisporic acid D. Organic Letters 8: 1669–1672.

Smith MM, Warren VA, Thomas BS, Brochu RM, Ertel EA, Rohrer S, Schaeffer J, Schmatz DM, Petuch BR, Tang YS, Meinke PT, Kaczorowski GJ, Cohen CJ. 2000, Nodulisporic acid opens insect glutamate-gated chloride channels: identification of a new high affinity modulator. Biochemistry 39: 5543–5554.

Stadler M, Wollweber H, Fournier J. 2004. host-specific species of *Hypoxylon* from France, and notes on the chemotaxonomy of the "*Hypoxylon rubiginosum* complex". Mycotaxon 90: 187–211.

Stadler M, Hellwig V. 2005. Chemotaxonomy of the *Xylariaceae* and remarkable bioactive compounds from *Xylariales* and their associated asexual stages. Recent Research Development Phytochemistry 9: 41–93.

Suwannasai N, Rodtong S, Thienhirun S, Whalley AJS. 2005. New species and phylogenetic relationships of *Hypoxylon* species found in Thailand inferred from the internal transcribed spacer regions of ribosomal DNA sequences. Mycotaxon 94: 303–324.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31: 21–32.

Triebel D, Persoh D, Wollweber H, Stadler M. 2005. Phylogenetic relationships among *Daldinia, Entonaema* and *Hypoxylon* as inferred from ITS nrDNA analyses of Xylariales. Nova Hedwigia 80: 25–43.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A guide to methods and applications (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). Academic Press Inc., San Diego, USA: 315–322.