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A molecular reappraisal of *Nimbospora* (Halosphaeriaceae, Microascales) and a new genus *Ebullia* for *N. octonae*

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ABSTRACT

Nimbospora is a genus in the Halosphaeriaceae with three species: *N. effusa* (the type species), *N. bipolaris* and *N. octonae*. All species have two-celled ascospores with a prominent sheath. A second type of appendage is present in two species, a single tuft of fibrillar appendages is present in *N. effusa* and two occur in *N. bipolaris*. *Nimbospora effusa* and *N. bipolaris* are morphologically similar, but there are major morphological differences in ascomatal morphology between *N. effusa*/*N. bipolaris* and *N. octonae*. In this study, we investigated the morphology of *N. effusa* and *N. octonae* and the phylogenetic relationships of the three *Nimbospora* species based on partial sequences of 18S and 28S rRNA genes. Bayesian analysis suggested that *Nimbospora* is not monophyletic. *Nimbospora effusa* groups with *N. bipolaris* in a well-supported clade, with *Naufragella spinibarbata* forming a sister group. *Nimbospora octonae*, however, clusters with *Haligena elaterophora* in a separate, well-supported clade. The ascomata of *N. octonae* differ from those of *N. effusa* and *N. bipolaris* by their thick peridium, and ascospores that lack equatorial tufts of appendages but possess polar and equatorial subulate appendages after the sheath is dissolved. Based on these characters and the correlating phylogenetic distance, the new genus *Ebullia* is established to accommodate *N. octonae*.

1. Introduction

Nimbospora Jørg. Koch is a genus in the Halosphaeriaceae, Microascales (Sordariomycetes, Ascomycota) with *N. effusa* Jørg. Koch as the type species (Koch 1982). Currently, there are three lignicolous species in *Nimbospora*, namely *N. effusa*, *N. bipolaris* K.D. Hyde & E.B.G. Jones (Hyde and Jones 1985) and *N. octonae* Kohlm. (Kohlmeyer 1985). Originally described from driftwood collected from a coastal area of Sri Lanka in the Indian Ocean, *N. effusa* is a typical member of the Halosphaeriaceae, with globose ascomata, catenophyses, thin-walled, deliquescing asci and hyaline ascospores with

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appendages. Ascospores of *N. effusa* are surrounded by a thick sheath, and a tuft of fibrillar appendages is attached to one side of the ascospore septum, which extends after the asci are dissolved (Koch 1982).

Nimbospora bipolaris is similar to *N. effusa* in morphology. Instead of having one tuft of fibrillar appendages at the spore equator, *N. bipolaris* has two tufts of appendages, one on each side of the central septum, and the sheath is constricted at the equator and less eccentric than in *N. effusa* (Read et al. 1993). Although an exosporic sheath is present around the ascospores of *N. octonae*, no tuft-like equatorial appendages occur. Instead, subulate appendages are present at the equatorial and polar positions of the ascospores after the sheath has deliquesced (Kohlmeyer 1985).

During our ongoing study of the diversity of marine fungi in Taiwan, all three known *Nimbospora* species were collected. A morphological study was initiated to re-examine the features of these species, especially the peridial wall structure based on paraffin sections. Because partial 18S and 28S rRNA genes were available for most species of the Halosphaeriaceae, these genes were consequently sequenced and analyzed by Bayesian inference or maximum likelihood to determine (1) if *Nimbospora* as currently delimited is monophyletic; and (2) the phylogenetic relationships between *Nimbospora* and other genera in the family.

2. Materials and methods

2.1. Collection, identification and isolation

Driftwood/trapped wood was collected in northeastern Taiwan. Wood samples were placed in large Zip-lock plastic bags and incubated at room temperature in the laboratory. Ascomata of *Nimbospora* species on wood were cut open with a razor blade under a SZ61 stereomicroscope (Olympus, Tokyo). Centrum material was transferred to a drop of sterile, natural seawater on a glass slide. The morphology of asci and ascospores was observed under a BX51 microscope (Olympus) and photographs made on a DP20 Microscope Camera (Olympus).

For isolation, a spore suspension of *Nimbospora* species was made by transferring centrum material to a drop of sterile, natural seawater on a sterilized glass slide. The spore mass was dispersed evenly in the drop of seawater with sterilized tweezers and identifications were confirmed by observation under a compound microscope. More sterile, natural seawater was added and dispensed onto the surface of a cornmeal seawater agar (CMAS) plate (Difco, Sparks, MD, USA) supplemented with 0.5 g/L each of penicillin G and streptomycin sulfate (BioShop, Burlington, ON, Canada). The plate was incubated at 25 °C for 1–3 days. Germinated single spores were picked up and transferred to fresh CMAS plates. The plates were incubated at 25 °C and cultures are deposited at the Bioresource Collection and Research Centre (BCRC), Hsinchu, Taiwan (ROC).

2.2. Section of ascomata

For sections of ascomata, pieces of wood with ascomata were cut out and fixed in FAA solution (5% formaldehyde and 5% glacial acetic acid in 50% ethanol) overnight at 4 °C. The fixed samples were washed three times in 50% ethanol. Samples were then dehydrated in a graduated t-butanol/ethanol/water series (10/40/50, 20/50/30, 35/50/15, 55/45/0, 75/25/0, 100/0/0, 100/0/0, in percentage by volume), and infiltrated gradually and embedded in paraffin. Paraffin sections (7 μm) were cut on a FRM-200P rotary microtome (Physitemp Instruments, Taipei), floated on water at 42 °C to relax compression and mounted on microscope slides. Dried sections were de-paraffinised and rehydrated through a graded series of ethanol. The sections were stained with 1% safranin O in 50% ethanol (10 s) and 0.5% Orange G in 95% ethanol (30 s). After washing and dehydration, each stained section was permanently mounted with a cover slip and Histokitt (Assistent, Sonheim/Rhön, Germany). Specimens were observed on the Olympus BX51 microscope and light micrographs were taken.

2.3. Molecular analysis

The isolates were grown on potato dextrose seawater agar plates (Difco) for at least 2 weeks at 25 °C. Mycelium was scraped off the agar surface and ground into powder in a mortar and pestle in liquid nitrogen. The DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used for genomic DNA extraction following the manufacturer's instructions. Extracted DNA was used directly for PCR reactions with the following ingredients: 0.2 μM of each primer (NS1/NS4, White et al. 1990; LROR/LR6, Bunyard et al. 1994), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$ and 1 U of Taq Polymerase (Invitrogen, São Paulo, Brazil). The amplification cycle consisted of an initial denaturation step of 94 °C for 5 min followed by 35 cycles of (i) denaturation (94 °C for 0.5 min), (ii) annealing (55 °C for 0.5 min) and (iii) elongation (72 °C for 0.5 min) and a final 11 min elongation step at 72 °C. The PCR products were analyzed by agarose gel electrophoresis and shipped to Tri-I Biotech, Inc., Taiwan, for purification and direct sequencing with the same primers.

Returned sequences were checked for ambiguity, assembled and deposited in GenBank (Table 1). These sequences and those from the GenBank were manually aligned in Se-Al v1.0a1 (Rambaut 1999). The alignments (TreeBASE accession no. 14728) were entered into BEAUti v1.7.2 for generation of XML files for Bayesian analysis in BEASTv.1.7.2 (Drummond and Rambaut 2007). Two data sets were analyzed (28S, 18S + 28S) with the following analytical settings: GTR, estimated base frequency, gamma + invariant sites, number of gamma categories set at 4, a strict clock with estimated evolutionary rate and normal rate distribution, the Yule process as the speciation model, with 20 million for 28S and 50 million generations for the combined 18S + 28S data sets, with parameters and trees sampled every 1000 generations. Convergence of the analyses was checked in Tracer v1.5 (Drummond and Rambaut 2007) and the effective sample size (ESS) of the parameter statistics >200 was ensured. The first 10% of the trees were treated as burn-in and discarded. A summary tree was produced using TreeAnnotator v1.7.2 (Drummond and Rambaut 2007) and viewed and edited in FigTree v1.3.1 (Rambaut 2009). For the combined 18S + 28S data set, a maximum likelihood bootstrap analysis was performed in Mega 5.2.2 (Tamura et al. 2011) with the following settings: 1000