15 *Neodeightonia*

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15.1 INTRODUCTION

15.1.1 Classification, Morphology, and Biology

The ascomycete genus *Neodeightonia* C. Booth belongs in the family Botryosphaeriaceae, order Botryosphaeriales. Members of the family Botryosphaeriaceae are best known as pathogens, saprophytes, and endophytes in a wide range of plant hosts.[1] Their occurrence and relevance as human pathogens is low in comparison to other clinical fungi, and only a few species such as *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.,[2,3] *Macrophomina phaseolina* Tassi (Goid.),[4,5] *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers,[4,6] and *Neodeightonia subglobosa* C. Booth[7] have shown the potential to cause disease to man.

The genus *Neodeightonia* was introduced by Booth (in Punithalingam 1969)[8] to accommodate a single species, namely *N. subglobosa*. von Arx & Müller (1975)[9] within their broad concept of the genus transferred this species to *Botryosphaeria*. Recently, the taxonomy and systematics of the genus *Botryosphaeria* sensu lato has been subjected to considerable revision.[10,11] In their work on the dark-spored telemorph genera in the Botryosphaeriaceae, Phillips et al. (2008)[11] reinstated *Neodeightonia* on account of its phylogenetic and morphological distinction from other genera in the Botryosphaeriaceae.

Thus, currently *Neodeightonia* is a very small genus comprising only two species, namely the type species *N. subglobosa* and *N. phoenicum* A.J.L. Phillips & Crous.[11] The last species was introduced by Phillips et al. (2008)[11] so to accommodate isolates obtained from palms (*Phoenix dactylifera* and *Phoenix canariensis*) and there is no evidence to date that it may incite any kind of clinical manifestation.

Neodeightonia subglobosa is characterized by having clavate, thick-walled bitunicate, eight-spored asci typical of the members of the Botryosphaeriaceae. Ascospores are distichous, the mature ones are brown, oval to broadly ellipsoidal, becoming one-septate, measuring 20–26 × 7–10 µm with a finely roughened surface. Its coelomycete anamorph has spherical to subglobose conidia which are initially hyaline and become light to dark brown when mature, aseptate, and measuring 9–12 × 6–9 µm.[8] In *N. phoenicum* conidia are ovoid to ellipsoid, with the apex and base broadly rounded, widest in middle to upper third, thick-walled, initially hyaline and aseptate, becoming dark brown and one-septate some time after discharge from pycnidia, with melanin deposits on the inner surface of wall arranged longitudinally giving a striate appearance to conidia, and measure 18.6–19.5 × 11.2–11.8 µm.[11] The teleomorph of *N. phoenicum* has not been found. Detailed morphological descriptions as well as microphotographs and illustrations of the genus and species can be found elsewhere.[8,10,11]

Neodeightonia subglobosa is a poorly studied species and virtually nothing is known regarding its biology, geographic distribution, and ecology. *Neodeightonia subglobosa* (as *Sphaeropsis subglobosa*) was first described from dead culms of *Bambusa* in Georgetown (Guyana) in 1879 and Belgium in 1882. Both states (anamorph and telemorph) of this species were described in 1969 on dead culms of *Bambusa arundinacea* in Sierra Leone. Since then the sexual state or teleomorph was never found again and there are only two reports published concerning this species. The first one deals with its occurrence as the cause of keratomycosis on a man who had poked a bamboo cane into his eye.[7] The second and most recent one describes the occurrence of this species as an

endophyte on herbaceous medicinal plants from India.[12] It is not clear if this species is a plant pathogen or merely a saprophyte that is able to grow on dead plant material. As occurs with several plant pathogens in the Botryosphaeriaceae,[1] it is likely that *N. subglobosa* is a plant pathogen that has an endophytic stage in its life cycle and that it is capable of causing diseases when the host is under stress. However, this assumption remains to be proven.

15.1.2 Clinical Features and Pathogenesis

As mentioned earlier *N. subglobosa* is a poorly known species and this applies also to the clinical environment. There is only a single case reporting this species as a human pathogen causing keratomycosis.[7] The patient was accidentally injured in the eye with a bamboo cane causing a shelving, penetrating corneal wound, which contained small slivers of bamboo. The splinters were removed and in agreement with the fungal sensitivity tests, a treatment with 2% clotrimazole eye drops was applied for 16 weeks. This resulted in the restoration of complete visual acuity. Nevertheless, 39 months after the end of the topical antifungal therapy the infection recurred and a keratoplasty had to be performed in order to remove the fungal mass that developed. The surgery was followed by topical steroid and clotrimazole therapy[7] and there was no subsequent relapse.

According to Kirkness et al.[7] fungal keratytis due to *N. subglobosa* is difficult to treat, especially when associated with a penetrating injury. These authors hypothesized that dark, thick-walled hyphae or brown conidia, which could be resistant to antifungal therapy, may have lain dormant in the cornea resulting in a recurrent infection.

Neodeightonia subglobosa is probably not a primary human pathogen and as in most coelomycetous fungi it is most likely an opportunistic human pathogen. In the coelomycetes causing opportunistic mycoses the infection occurs most frequently by implantation of the fungus from plant material (or soil) through abrasions, lacerations, puncture wounds, or other traumas rather than by inhalation of spores.[13] These fungi may also be of concern to immunocompromised patients, such as bone marrow and organ transplant recipients, cancer patients, among others.[13]

15.1.3 Diagnosis

15.1.3.1 Conventional Techniques

As for other mycoses the hispathological identification of hyphae is elemental to confirm a fungus as the causal agent of the disease. Although the pigmentation and shape of hyphae and the presence or absence of septa can give an idea of the identity, a fungal culture is required to accomplish an accurate and reliable diagnosis of the etiological agent.[13,14] The identification of fungi, unlike other important pathogens such as bacteria or viruses, relies mainly on morphological criteria. Most specifically fungal identification is based on the morphology of the reproductive structures.

Coelomycetous fungi generally display a moderate to rapid growth rate on a variety of routine culture media and are not particularly difficult to recover from excised material. The main problem lies in promoting sporulation in order to obtain the diagnostic reproductive structures necessary for identification of the isolates. Apart from the considerable amount of time required, particularly for pycnidial species (up to months in some strains), it is also necessary to employ a medium upon which these pycnidia will develop.[13]

The culture of Coelomycetes on sterilized plant tissue is deemed to produce conidiomata more representative of those in nature. It is known that culture on nutrient-rich synthetic media often results in atypical characteristics and that sporulation may be hindered or absent on sugar-rich media.[13,15] Our experience with cultures of botryosphaeriaceous fungi, such as *Neodeightonia*, has shown that the addition of sterilized plant material (e.g., pine needles, twigs of oak, poplar, or other plants) on water agar, or half-strength potato dextrose agar, encourage the production of conidiomata. Another medium that is frequently used is oatmeal agar.[11,16]

As mentioned above, the identification of fungi is based on their morphology. Despite the fact that the sexual stages (telemorphs) are the baseline of fungal taxonomy and nomenclature, medical mycologists are generally more familiar with the asexual stage or anamorph, which is the stage most frequently found in cultures of clinical isolates.[14] In the case of botryosphaeriaceous fungi the teleomorph is rarely found in nature and it is highly improbable that sexual fruiting bodies (pseudothecia) will be seen in cultures of clinical isolates. Although Punithalingam (1969)[8] reported that *N. subglobosa* is a homothallic species and its teleomorph forms in culture, we have not observed that in our work. In fact, in the closely related species *N. phoenicum* our isolates also did not form the teleomorph in culture, even after long periods of incubation (>3 months).[11]

Species identification in the Botryosphaeriaceae has relied heavily on morphological characters of the anamorph, including size, shape, color, septation, wall thickness, and texture of conidia. However, some morphological characters exhibit extensive plasticity. Thus, size ranges of conidia of different species overlap, while age and state of maturity affect conidial pigmentation and septation. Morphological characters can also be influenced by the substrate on which the fungus is growing.[1,17,18] Because species may differ in minor morphological features, identification can be a difficult task for someone who is not familiar with these fungi.

The genus *Neodeightonia* is phylogenetically related to *Diplodia* and *Lasiodiplodia* and has intermediate morphological features between both genera.[11] Conidia are initially hyaline and aseptate becoming brown and one-septate (in some cases) with age, characters that are common to both *Diplodia* and *Lasiodiplodia*. Punithalingam[8] referred to a germ slit in the conidia of *N. subglobosa*. Crous et al.[10] suggested that this is in fact a striation on the conidial wall, and that more than one could occur per conidium, a feature that was confirmed in a later study.[11] Moreover, Phillips et al.[11] described a new species in *Neodeightonia*, namely *N. phoenicum*, which has

clear striations in the conidial wall. Such striate walls are a typical feature of *Lasiodiplodia* species suggesting an affinity to this genus. Nevertheless, *Neodeightonia* can be distinguished from *Lasiodiplodia* by the absence of pycnidial paraphyses which are also not present in *Diplodia*. Thus, conidial striations distinguish *Neodeightonia* from *Diplodia*, and the absence of pycnidial paraphyses distinguishes it from *Lasiodiplodia*.

Identification to species level is not as complicated as in other genera of Botryosphaeriaceae because the genus is quite small currently comprising only two species, that is, *Neodeightonia subglobosa* and *N. phoenicum*, which are easily distinguished on the size, shape, and septation of the conidia. Conidia of *N. subglobosa* are spherical to subglobose, aseptate, and smaller than the conidia of *N. phoenicum* which are oval to ellipsoid and have one transverse septum.

15.1.3.2 Molecular Techniques

Keratytis is currently recognized as the most common fungal infection of the eye, with some authors reporting up to 16%–37% of keratitis cases related to a mycotic aetiology.[19,20] Early, rapid, and accurate identification of the fungal species is important in order to guide the selection of appropriate antifungal therapy and is essential for an effective treatment of ocular infections such as fungal keratytis.[19–22]

Diagnosis of fungal infections depends on recovery of fungi from culture of clinical specimens, and their identification requires the presence of reproductive structures. Clinical diagnosis of these ocular infections is confirmed by obtaining intraocular (aqueous or vitreous) specimens or corneal scrapings. However, standard microbiological tests (Gram and Giemsa stains and culture) are positive in only 50%–80% of keratitis cases.[19,23,24] Even in the cases where culture tests are positive an identification of the fungal species can be difficult to obtain. The culture-based phenotypic methods can be time consuming and laborious, may initially be nonspecific, and require considerable expertise for correct morphological identification of less common or unusual fungi. Additional drawbacks of conventional morphological identification are the inability of some cultures to sporulate, or cultures exhibiting atypical morphology resulting in the impossibility to identify the species or even in misidentifications.[25–27] In fact, the occurrence of non-sporulating moulds seems frequent in cultures derived from ocular infections.[25,27]

For *Neodeightonia* isolation and morphological identification of cultures from clinical specimens has been successful in the single case reported to date.[7] Nevertheless, one cannot discard the chance that in some cases retrieving cultures will not be possible and some cultures/strains may not be able to sporulate thus hindering the identification. Also, even if successful isolation is accomplished the sporulation process (development of conidiomata and conidia) may take 2–3 weeks to occur,[11] a time frame that is not clinically useful.

Molecular methods for identification of pathogenic fungi have been validated for use in clinical settings in order to overcome the shortcomings of traditional morphological identification mentioned previously. The field of medical mycology has definitively embraced molecular methods of identification/detection of fungi and these methods are being increasingly applied.[19–29] Molecular methods are rapid with a turnaround time of about 24 h from the time of DNA extraction, yield results that are objective with data portable between laboratories, and despite the higher cost could be more economical in the long run.[19–29] These methods, especially the polymerase chain reaction (PCR)-based ones, can be applied to cultures for which the phenotypic approach has been found to be ineffective, and offer the additional advantage of eliminating the need for isolation of cultures as they can be applied directly to clinical samples.[19–27]

A myriad of DNA-based identification methods is available and these are being increasingly employed in clinical laboratories. These nonsequence-based molecular methods include the commercially available GenProbe assay, methods based on the polymerase chain reaction such as single-step PCR, RAPD-PCR, rep-PCR, nested PCR, PCR-RFLP, PCR-EIA, and more recent microassay-based, Luminex technology-based, and real-time PCR-based methods. Great variation in assay complexity, targets, and detection methods can be found, and many of these methods have not been widely used or rigorously validated.[29] Of the above-mentioned methods rep-PCR and MSP-PCR have proven useful for differentiating *Neodeightonia* species,[30] but their applicability in clinical settings has not been evaluated.

At present, the "gold standard" for fungal species identification is DNA sequence analysis. This method is based on PCR amplification of a selected region of genomic DNA, followed by sequencing of the resulting amplicon. The obtained sequence can then be used to perform searches against a nucleotide sequence database library.[20,22,25,28]

The success of this strategy depends largely on the choice of the appropriate locus. The gene target must be orthologous, have a high level of interspecific variability combined with low levels of intraspecific variability, and should not undergo recombination. Additionally, it must be easy to amplify and sequence and the amplicons should be within the size range obtainable with the most commonly used automated DNA sequencers (about 600–800 bp).[14,28,29]

The ITS region (noncoding sequence interspaced among highly conserved fungal rDNA genes) complies with most of these requirements since this region can be reliably amplified for most fungi, is conserved, is present as multiple copies in the fungal genome, yields sufficient taxonomic resolution for most fungi, and has the additional advantage that the GenBank (http://www.ncbi.nlm.nih.gov), European Molecular Biology Laboratory nucleotide sequence database (http://www.ebi.ac.uk/embl/), and DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/) contain a large number of sequences from this locus, enabling a ready comparison of the sequence from an unknown isolate.[14,28,29]

There is considerable consensus regarding the use of ITS sequencing in the identification of fungi. Also, the International Subcommission on Fungal Barcoding has proposed the ITS region as the prime fungal barcode or the default region for species identification. The main disadvantages

regarding the use of ITS is the inability of this region for differentiation of species complexes and failure to distinguish between closely related species or cryptic species.[14,28,29] In these cases, it may be necessary to use more variable gene regions. The locus to be used will depend on the fungal group in question. For example, in the Botryosphaeriaceae when the ITS region fails to differentiate species complexes or cryptic species the locus of choice has been the elongation factor 1-alpha which has shown successful results.[31]

Regarding *Neodeightonia* the molecular identification of species can be based solely on the ITS sequence. ITS sequences of *N. subglobosa* and *N. phoenicum* are sufficiently different from one another to account for a reliable identification at species level. In the course of recent studies dealing with the genus *Neodeightonia* and other genera in Botryosphaeriaceae[10,11] sequences of the 18S and 28S rDNA (D1/D2 variable region) and ITS region, as well as the elongation factor 1-alpha and beta-tubulin became available for authentic and ex-type cultures of *N. subglobosa* and *N. phoenicum*. This fact is highly relevant since the success of the DNA sequence-based identification depends on the reliability of sequences deposited in reference databases.

PCR-based culture independent methods have been developed and applied for the detection of fungi causing keratomycosis directly on ocular specimens.[21,24] Also, through the use of species specific oligonucleotide primers or probes this approach has been used to detect a particular species or group of species without the need for DNA sequencing.[32] To date, no specific primers or oligonucleotide probes have been developed for species of *Neodeightonia* and thus if molecular detection methods are to be applied to *Neodeightonia* species these will have to depend (for now) on the PCR amplification and sequencing of the ITS region.

15.2 METHODS

15.2.1 Sample Preparation

The first and probably the most important step in any molecular detection method is the isolation of DNA from the fungal culture or directly from the clinical specimen. Although for PCR-based methods the amount of DNA required is very small it is important to use DNA extraction procedures that guarantee a sufficient quantity of DNA and simultaneously that it is not contaminated with PCR inhibitory agents. A good DNA extraction method is crucial for PCR detection to avoid the possibilities of false negative results. Also, strict precautions must be taken throughout the whole procedure, from collection and processing of samples to DNA extraction and PCR amplification in order to avoid contaminations and consequently false positive results.

Clinical diagnosis of keratomycosis is confirmed by obtaining intraocular (aqueous or vitreous) specimens or corneal scrapings.[19–21,24,25] These samples can be used to inoculate culture media in order to isolate the fungus responsible for the infection. As soon as colonies start to develop DNA can be extracted by a number of existing methods, including commercial kits which are usually faster than other approaches.[16,19,20,24–27,32] In a faster approach the DNA can be obtained directly from the ocular samples via the same DNA extraction procedures used for fungal cultures or others developed specifically for human tissue samples.[20,22,32] By eliminating the isolation and cultivation procedure this last approach greatly reduces the time necessary for the molecular diagnosis of the causal agent.

Procedure

1. Inoculate cultures onto potato dextrose agar (Difco) and incubate at 25°C. After 1 week the colony is scraped from the agar surface, frozen in liquid nitrogen and ground in a porcelain mortar. As an alternative, cultures can be grown in potato dextrose broth.
2. Genomic DNA can be extracted from the ground mycelium using any DNA isolation protocol. In our lab we have good results in terms of yield and purity of DNA samples following the method described by Alves et al. (2004).[16]

15.2.2 Detection Procedures

Once DNA is obtained it is necessary to perform a PCR amplification of the ITS region. The ITS can be amplified easily using "universal" fungal primers in the vast majority of fungi. Our experience shows that with *Neodeightonia* (and Botryospheriaceae in general) the primer sets ITS1/ITS4 or ITS5/ITS4[33] result in good amplification of the target region resulting in amplicons with a size of about 500–600 bp.[11,16,31] As an alternative the ITS1 forward primer can be combined with the primer NL4[34] which anneals within the 28S rDNA gene resulting in a larger amplicon (approx. 1200 bp) that includes the ITS region plus the D1/D2 variable region of the 28S rDNA gene.[35] The PCR mixtures and amplification conditions have been described elsewhere.[11,16,35] In some cases where amplification is weak or unsuccessful, good results are usually obtained by performing a second PCR using as template the first PCR amplification.[36] Another approach involving a nested or semi-nested PCR can be performed. Thus, for example, an initial amplification would be performed using the primer set ITS1/NL4 and in the second PCR the primer set used would be ITS1/ITS4. This kind of approach is frequently used and is known to increase the sensitivity of the PCR detection.[19] Finally, from our experience, the addition of 5% DMSO to the PCR amplification reaction is useful to help the amplification of some difficult templates and also to increase reproducibility.[11,31,35,36]

The nucleotide sequence of the ITS region is then obtained using standard automated DNA sequencing procedures. The complete sequences of the ITS region are read and edited using available software such as Chromas 1.45 (http://www.technelysium.com.au/chromas.html), FinchTV 1.4.0 (http://www.geospiza.com/products/finchtv.shtml), or others. The sequences must be checked manually and nucleotide

arrangements at ambiguous positions clarified using both primer direction sequences. The final step in the identification procedure consists in performing a sequence similarity search of the retrieved sequence against nucleotide sequences database such as GenBank using the BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). The outcome of this similarity search must be interpreted with caution due to the well known problems with the reliability of the ITS sequences deposited in the reference databases (e.g., GenBank/EMBL/DDBJ).[28,37] Errors in fungal sequences within GenBank, the most widely used database, have been found to be as high as 20%.[37] However, in the case of *Neodeightonia* this task is facilitated since sequences from authentic, ex-type and well-characterized species are available in GenBank.

Procedure

1. Prepare a PCR mixture (50 µL) containing 1× PCR buffer with $(NH_4)_2SO_4$ (MBI Fermentas, Vilnius, Lithuania), 3 mM $MgCl_2$, 200 mM of each nucleotide, 15 pmol of primer ITS1, 15 pmol of primer ITS4, 1 U of *Taq* DNA polymerase (MBI Fermentas), and 50 ng of template DNA.
   Note: The forward primer ITS5 can be used as an alternative to primer ITS1.
2. Perform the PCR amplification in a thermocycler using the following conditions: initial denaturation of 3 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, and a final extension period of 10 min at 72°C.
3. Purify the PCR amplicons before sequencing. In our lab we have good results with the Jet Quick PCR Product Purification Spin Kit (Genomed, Löhne, Germany) but virtually any PCR product purification kit can be used.
4. Prepare cycle sequencing reactions (20 µL) containing 30–90 ng DNA template, 3.2 pmol of primer, 4 µL BigDye? Terminator from the ABI PRISM? BigDye, and Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems).
5. Remove excess dye terminator by mixing the reaction with 2 µL of 3 M sodium acetate (pH 4.6) and 50 µL of 100% ethanol. Precipitate for 20 min at room temperature and centrifuge for 20 min at 16,000× *g*. Discard the supernatant and wash the pellet with 250 µL of 70% ethanol. Dry the pellet in a heat block at 90°C for 1 min.
6. Sequence in an automated DNA sequencer such as the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

15.3 CONCLUSION AND FUTURE PERSPECTIVES

The incidence and prevalence of invasive mycoses due to opportunistic fungal pathogens has increased significantly over the past 2 decades. The field of medical mycology has become an extremely challenging study of infections caused by a wide and taxonomically diverse array of opportunistic fungi. These opportunistic mycoses pose considerable diagnostic and therapeutic challenges and a rapid, accurate diagnosis is essential for the initiation of targeted antifungal therapy.

Given the general lack of knowledge about *N. subglobosa* in clinical settings and the aforementioned problems with traditional morphological identification of fungi it is possible that infections caused by this species may have previously passed unnoticed due to inadequate diagnosis. The clinical significance of *N. subglobosa* is poorly understood. However, as emphasized by Kirkness et al. (1991)[7] this species should be considered in ocular infections as a result of injuries caused by plant materials. This fungus should be particularly considered in tropical and subtropical countries where incidence of keratomycosis is higher than in temperate regions.[25]

Although *Neodeightonia* species are not especially difficult to identify based on morphological characters, they require considerable expertise of the taxonomy of the Botryosphaeriaceae. Because this group of fungi is not frequent in clinical settings this may constitute a problem when trying to obtain a correct identification. In this respect, molecular methods have the advantage that no taxonomic expertise is required. Moreover, the molecular detection procedure can be performed directly on the clinical specimen without the need to isolate the fungus, which decreases the time needed for the diagnosis and consequently for the initiation of proper antifungal therapy.

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