A new endophytic species of Neostagonospora (Pleosporales) from the coastal grass Spinifex littoreus in Taiwan

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| Complete List of Authors: | Yang, Jun-Wei; National Central University  
Yeh, Yu-Hung; National Central University  
Kirschner, Roland; Department of Life Sciences, National Central University, Taiwan |
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A new endophytic species of *Neostagonospora* (Pleosporales) from the coastal grass *Spinifex littoreus* in Taiwan

Jun-Wei Yang, Yu-Hung Yeh, and Roland Kirschner

**Jun-Wei Yang, Yu-Hung Yeh, and Roland Kirschner.** Department of Life Sciences, National Central University, Jhongli District, Taoyuan City 32001, Taiwan (R.O.C.).

**Corresponding author:** Roland Kirschner (e-mail: kirschner@ncu.edu.tw).
Abstract: A new species of Neostagonospora associated with Spinifex littoreus was found. Endophytic isolates remained sterile, but a specimen collected on living leaves could be used for morphological characterization and cultivation. The ITS sequences from the cultures as well as from direct total DNA extraction from the plant indicated conspecificity and a broader geographic distribution than the single field collection, but yielded low support on the genus level in phylogenetic analyses. Analysis of the RPB2 sequences, however, sufficiently supported a placement of the species in Neostagonospora. The shape of conidiogenous cells as well as numbers of septa and sizes of conidia are useful characters for distinguishing Neostagonospora species.

Key words: coastal dunes, coelomycetes, culture-dependent, culture-independent, Dothideomycetes.

Introduction

Endophytes are microorganisms that live asymptotically within plant tissues (Narayan et al. 2006). Some endophytes may transform into pathogens causing plant diseases, whereas others may become saprobic decomposers after senescence and death of the plant. In other cases, however, endophytes benefit their host plants by producing a plethora of substances that provide protection and improve the survival of
the plants (Waller et al. 2005). These features indicate that endophytes play an important role in ecological communities.

Most of studies on endophytes have been conducted using a culture-dependent approach. It can, however, be expected that the actual diversity of endophytes colonizing plants is as high as or higher than that suggested by cultivation-based approaches (Zimmerman and Vitousek 2012). The culture-independent approach relies on DNA sequences obtained from the plant sample. The culture-dependent approach usually yields a high proportion of sterile mycelia without characteristics suitable for morphological identification. Because of the lack of DNA sequence data for ca. 80% of the described fungal species (Hawksworth 2004), attempts often fail to identify species with DNA sequences. In this study, we combined the culture-dependent, culture-independent and field collection approaches to investigate the diversity of endophytes and to identify species which could not be identified with a single approach alone.

On the coast of Taiwan, Spinifex littoreus (Poaceae) grows on sandy beaches and dunes. To propagate and overcome sand burial, the species produces vertical and horizontal rhizomes and stolons that contribute to the formation of dunes (Maun 2009). Besides its importance for stabilizing and protecting coastal areas, S. littoreus has also been shown to have anti-inflammatory properties (Yogamoorthi and Priya
2006). Little, however, is known about the organisms associated with this plant.

Through culture-dependent and culture-independent approaches and morphological study of a single specimen gathered in the field, a species was identified as a member of *Neostagonospora*, but was not identical to any of the previously described species of that genus.

The genus *Neostagonospora* was introduced by Quaedvlieg et al. (2013) and is characterized by immersed, pycnidial conidiomata, phialidic conidiogenous cells with a periclinal apical wall-thickening, and hyaline, septate conidia. The genus currently includes two species and in this study, an additional species, *Neostagonospora spinificis* is proposed based on molecular analyses and morphological characteristics.

**Materials and Methods**

Altogether, 21 individuals of *Spinifex littoreus* were collected in four locations on sandy sites of the northern and eastern coast of Taiwan, i.e. Taipei City, Taoyuan, Miaoli and Yilan Counties from 2011 to 2014. Plants were removed with a trowel, individually placed in bags, returned to the laboratory and kept at 4 °C until further processing within 48 hours after sampling. Seventeen of these plants were used exclusively to study endophytes in pure culture. These plants were washed and divided into root, stem, leaf sheath, and leaf lamina before being surface sterilized as
in Yeh and Kirschner (2014). Three parts of additional plants collected in 2014 were cut transversally into six fragments. Three of the fragments were used for the culture-dependent approach and the remaining three for the culture-independent one. An additional living plant showing leaf spots was collected in 2013.

For the isolation and cultivation of endophytes, 1.3% malt-extract agar was used for culturing from surface-sterilized plant parts. Extraction of DNA and PCR amplification of internal transcribed spacer (ITS) and large subunit (LSU) ribosomal DNA regions, and gel electrophoresis were done as in Yeh and Kirschner (2014). For the RNA polymerase II second largest subunit (RPB2) gene, PCR amplification conditions were as in Quaedvlieg et al. (2013). The ITS region was amplified with primers ITS1F and ITS4, the partial LSU ribosomal DNA with primers NL4 and NL1, and the partial RPB2 gene with primers fRPB2-5F and fRPB2-414R (White et al. 1990, O'Donnell 1993, Quaedvlieg et al. 2013). Sequencing of PCR amplicons was done by Mission Biotech (Nankang, Taipei) with the same primers for the PCR. To culture from the leaf spots, leaves with inconspicuous lesions were cut transversally in order to open the conidiomata. Conidia from the opened conidiomata were aseptically transferred with a flamed acupuncture needle to corn meal agar with 0.2% chloramphenicol. Then the specimen was dried and deposited in the Natural Museum.
of Natural Science, Taichung, Taiwan (TNM). The culture was deposited in the Bioresource Research and Collection Centre, Hsinchu, Taiwan (BCRC).

Total genomic DNA was extracted from surface-sterilized plant fragments with Genomic DNA Spin Kit (Plant), according to the modified manufacturer’s protocol (Bioman Scientific Co., Ltd., Taiwan). The primer pair ITS1F/ITS4 was used for amplification of the ITS rDNA (Gardes and Bruns 1993, White et al. 1990). The PCR products were separated on a 2.0% agarose gel and the band of interest was excised from the gel and purified with the Clean/Gel Extraction Kit (BioKit Biotechnology Co., Ltd., Taiwan). The purified products were ligated into the pJET1.2/blunt Cloning Vector and then recombinants were transformed into *Escherichia coli* DH5 alpha competent cells with CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Inc., USA). The positive transformants of ITS rDNA libraries were screened by PCR amplification of inserts using the pJET1.2 forward/ pJET1.2 reverse sequencing primer pair. PCR amplification products containing the correct size of insert were digested with 10 U of restriction enzyme *Msp I* µl⁻¹ for 1 h at 37 °C. The digested DNA fragments were separated on 2.0% agarose gels and the analyses of restriction fragment length polymorphism (RFLP) patterns were performed manually. The different PCR amplification products were sequenced by Mission Biotech (Nankang, Taipei).
All DNA sequences were edited using CodonCode Aligner version 4.0.1 (CodonCode Corporation, USA). DNA sequences generated in this study were deposited in the DNA Data Bank of Japan and in GenBank (Table 1). The following ITS rDNA sequences of the species treated in this paper were deposited in GenBank: KP676041 and KP676042 obtained by the culture-dependent approach, KP676043 and KP676044 by the culture-independent approach, and KP676045 from a culture derived from leaf lesions. A partial nuLSU rRNA gene sequence was also obtained from this culture and deposited in GenBank (KP676046). The ITS sequences were useful for comparison between species, but not for phylogenetic resolution (FIGURE 2). Following Quaedvlieg et al. (2013), RPB2 gene sequences were also generated and deposited in DDBJ for the culture derived from leaf lesions (LC055104) and cultures Z0809 (LC055105) and Z1104 (LC055106). Taxon sampling was based on a recent phylogenetic treatment of the Phaeosphaeriaceae (Quaedvlieg et al. 2013). Alignments of the RPB2 gene sequences were produced with the default options of MUSCLE implemented in MEGA6 (Tamura et al. 2013) without manual editing and after trimming the alignment block to a size of 310 characters. A maximum likelihood analysis was done on the alignment block using the Kimura 2-parameter model, gamma distributed with invariant sites, complete deletion of gaps, and 1000 bootstrap replicates. The tree shown in FIGURE 1 was rooted with Dothistroma pini Hulbary.
(Mycosphaerellaceae, Capnodiales). The alignment file has been deposited in TreeBASE (number 17770). In addition, the ITS sequences were also compared with other species using the NCBI Nucleotide BLAST tool.

Description of micromorphology is based on dry leaf material. The samples were sectioned by hand and mounted in 5–10% (w/v) aqueous KOH solution and 1% phloxine. The sections were examined at 1000x magnification with an Olympus CX41 light microscope. The statistical treatment of measurements was done with 30 measurements of the conidia which are given as mean value ± standard deviation with extreme values in brackets. Drawings were made using scaled paper and processed with Adobe Photoshop.

Results

All three approaches used in this work resulted in the identification of a species of *Neostagonospora*. Three strains of this species had identical RPB2 gene sequences, but these strains did not sporulate in culture and could not be compared morphologically with the known species. In the phylogenetic analysis, sequences derived from the new species clustered within the Phaeosphaeriaceae of the Pleosporales (FIGURE 1). On the other hand, the five ITS sequences of the new species formed a well supported clade, but the support for the genus was low.
(FIGURE 2). The infraspecific ITS sequence variation for the new species was 0–1 positions. When comparing ITS sequences exceeding 400 positions with their most similar matches from BLAST searches, sequences of the new species differed for 24–25 positions (4%) from those of *N. caricis* and for 32–33 positions (6%) from those of *N. elegiae*. According to the phylogenetic tree based on the RPB2 gene, “*Septoria*” *arundinacea* CBS 133.68 probably belongs to *Neostagonospora*. Its ITS sequence differed for 35–36 positions (6%) from those of the undescribed taxon. Species of the next most closely related genus belonged to *Parastagonospora*. The ITS sequences of these *Parastagonospora* species differed for at least 39 positions (7%) from those of the *Neostagonospora* species. In addition to molecular data, the morphological characteristics of the new species were different from those of the other *Neostagonospora* species. In contrast to the high morphological similarity between the new fungus from *S. littoreus* and *Ascochyta* species, their ITS sequence similarity was low (less than 90%). Based on the congruent results of the three approaches used in this work, a new species of *Neostagonospora* is proposed.

**Taxonomy**

*Neostagonospora spinificis* R. Kirschner, Jun-W. Yang and Yu Hung Yeh, sp. nov.

FIGURE 3
Index Fungorum IF550947

**Type:**—Taiwan, Miaoli County, Zhunan Township, sand beach north of Dragon and Phoenix Port (24.7017°N, 120.8589°E), on living leaves of *Spinifex littoreus*, 30.03.2013, R. Kirschner 3867 (TNM, holotype!). Ex-type strain: Bioresource Collection and Research Centre Taiwan (BCRC) FU30120. ITS rDNA sequence: GenBank KP676045. LSU rDNA sequence: GenBank KP676046. RPB2 gene sequence: DNA Data Bank of Japan (DDBJ) LC055104.

**Etymology:**—Derived from the host genus, *Spinifex*.

*Conidiomata* completely immersed, filling the substomatal chamber, forming globose pycnidia, up to 100 µm diam.; wall formed of one layer of pale brown textura angularis. *Conidiophores* reduced to conidiogenous cells that are broadly compressed ampulliform, brown, smooth, 5–8 µm wide and 2–4 µm high; slightly tapering at apex, with apical wall-thickening. *Conidia* hyaline, smooth, thin-walled, narrowly ellipsoidal or almost cylindrical, rarely slightly narrowing to the apex, apex subobtuse, base truncate, 1-septate at the median, measuring (17–)19–21(–24) × (3–)3.5(–4) µm (n = 30, av. ± SD: 19.7 ± 2.04 × 3.8 ± 0.4 µm). Habitat, living leaf blades of *Spinifex littoreus*, associated with green tissues or inconspicuous yellow discoloration around a pale black spot. Hitherto known only from Taiwan.
Discussion

When studying the fungal biodiversity of sand dune soil, Prenafeta Boldú et al. (2014) found that the culture-dependent approach favors easily cultivable saprobic fungi, whereas the fungi revealed by the DNA-based approach depended on the selected primers, and the field collections revealed macrofungi that were not detected by the other two approaches. Both the culture-dependent and culture-independent approaches yield quick and relatively season-independent insights into the diversity of fungi associated with a plant species by counting different operational taxonomic units (OTUs) based on different colony morphologies in culture and/or different DNA sequences. The species found with these two approaches differ considerably with only few species being recovered by both approaches (Gao et al. 2005). For a large proportion of these OTUs, however, further progress towards characterization and identification of species is not possible with these methods, because many cultures remain sterile or form in vitro-artifacts. In addition, ca. 80% of the described species lack DNA data (Hawksworth 2004). In order to overcome these obstacles, the traditional approach of field collection with morphological characterization remains indispensable. Field collection, however, has the disadvantage that morphologically recognizable specimens might be present only at a certain place and time. As an example, the fungus described in this paper was detected several times using the
culture-dependent as well as culture-independent approaches, but only once as traditional field specimen. In all approaches, the fungus was detected only in the leaves, not from stems or roots, which indicates a high specificity towards leaf tissue. Since a culture was successfully isolated from the field specimen, the ITS and RPB2 gene sequences that were obtained from the sterile cultures can be linked to the new species and a morphological description can be presented.

Based on its morphology, particularly on the one-septate conidia, the fungus was first tentatively identified as a species of *Ascochyta*. Among the accepted species of *Ascochyta* occurring on Poaceae, however, shapes and sizes do not match with those of the species from *S. littoreus* (Mel'nik 2000; Punithalingam 1979). The *Ascochyta* species most similar with respect to conidium size have low genetic similarity to the species with available DNA data (*Didymella exitialis* (Morini) E. Müll.). The species lacking DNA data differ by having a proven *Didymella* teleomorph (*D. graminicola* Punith.), or by having apically narrowing conidia (*A. arundinariae* Tassi, *A. hordeicola* Punith.), and minor differences of sizes of conidia or pycnidia (Punithalingam 1979). In addition, when the sequences from *N. spinificis* are compared with those available from *Ascochyta* species that exceed 400 positions, the similarity was less than 90%. *Ascochyta* is a polyphyletic genus within the Pleosporales, but the type species and close relatives are connected to *Didymella* in
the Didymellaceae (De Gruyter et al. 2009), whereas *Neostagonospora* phylogenetically belongs to the Phaeosphaeriaceae (Quaedvlieg et al. 2013).

According to Sutton (1980), conidiogenous cells of *Stagonospora* species in the former, broad sense, are holoblastic (i.e. without apical wall-thickenings), occasionally annellidic, and have conidia with at least two septa. In comparison, the species on *S. littoreus* forms conidiogenous cells with apical wall-thickenings and one-septate conidia. For morphological reasons, the fungus isolated from *S. littoreus* cannot be confused with the species formerly described in *Stagonospora*.

The placement of a strain designated as “*Septoria* arundinacea” by Quaedvlieg et al. (2013) in the *Neostagonospora* clade is confirmed in our study, but further data about this strain are lacking. If new strains with the same DNA sequences are isolated in the future and can be characterized morphologically, they could be placed within *Neostagonospora*.

The two known species of *Neostagonospora* were described only from culture, which might cause some artifacts, whereas the description of *N. spinificis* is based on material collected in nature. Unfortunately, the original specimens from field collections used by Quaedvlieg et al. (2013) were not preserved and details about the ‘symptomatic leaves’ indicating whether the fungi were rather parasitic or saprobic were not presented. *Neostagonospora spinificis* differs from the known species by at
least 24 different positions (4%) of the ITS region. Morphologically, *N. spinificis* and *N. caricis* differ from *N. elegiae* by their one-septate conidia, whereas conidia of *N. elegiae* are (0–)3-septate. *Neostagonospora spinificis* and *N. caricis* can be distinguished by the length of their conidia, i.e. no more than 19 µm in *N. caricis* and mostly no less than 19 µm for *N. spinificis*. Furthermore, the compressed shape of the conidiogenous cells of *N. spinificis* also differs from the ampulliform conidiogenous cells of the other two species. The deviating shape of conidiogenous cells as well as number of cell layers of the pycnidium (one in *N. spinificis*, more than one in the other species) are characters that vary inter- as well as infraspecifically within the same genus (Punithalingam 1979, Sutton 1980).

**Acknowledgements**

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**References**


**TABLE 1.** Origins and accession numbers of DNA sequences obtained for *Neostagonospora spinificis* from leaves of *Spinifex littoreus* in Taiwan.

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* CD=culture-dependent, CI=culture-independent, FC=field collection

**DDBJ=DNA Data Bank of Japan**
Legends

FIGURE 1. Maximum Likelihood tree showing estimated relationships of *Neostagonospora spinificis* among Pleosporales based on RPB2 gene sequences, the names given with quotes follow Quaedvlieg et al. (2013). Bootstrap values above 50% (1,000 replicates) are indicated at the nodes. The tree was rooted with *Dothistroma pini*.

FIGURE 2. Maximum Likelihood tree showing estimated relationships of *Neostagonospora spinificis* among Pleosporales based on ITS sequences, the names given with quotes follow Quaedvlieg et al. (2013). Bootstrap values above 50% (1,000 replicates) are indicated at the nodes. The tree was rooted with *Vrystaatia aloecola*.

FIGURE 1.
250x250mm (300 x 300 DPI)
Figure 2

274x218mm (300 x 300 DPI)