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Pezicula neosporulosa sp. nov. (Helotiales, Ascomycota), an endophytic fungus associated with *Abies* spp. in China and Europe

Zhilin Yuan [a,*], *Gerard J.M. Verkley* [b]

[a] *Institute of Subtropical Forestry, Chinese Academy of Forestry, Fuyang, 311400, Zhejiang Province, PR China*
[b] *CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584CT, Utrecht, The Netherlands*

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ABSTRACT

A new species of *Pezicula*, *P. neosporulosa* associated with *Abies alba* in the Netherlands and *A. beshanzuensis* in China is described, illustrated and compared to its sister species *P. sporulosa*, *P. cinnamomea* and *P. eucrita*. Morphologically, *P. neosporulosa* is shown to be similar to *P. sporulosa* both in sexual and asexual sporulating structures. However, from China the species is only known from endophytic isolates, and sporulating structures were never obtained in them. In contrast to the three sister species, the new species does not produce microconidia directly from ascospores or from macroconidia, but only from conidiophores in conidiomata. Moreover, the colony appearance is highly variable in endophytic isolates. Bayesian, maximum parsimony and maximum-likelihood phylogenetic analyses based on four unlinked loci (internal transcribed spacer, RPB2, TEF-1α and β-tubulin) in concatenated datasets confirmed that all isolates in *P. neosporulosa* were well separated from closely allied species with high bootstrap value and posterior probability. Taken together, the molecular and morphological evidence supports the introduction of *P. neosporulosa* as a novel taxon.

1. Introduction

Pezicula Tul. & C. Tul. nom. cons., is a genus of inoperculate discomycetes belonging to the family Dermateaceae (order Helotiales). Most *Pezicula* species are known to sporulate on dying bark, and in numerous fungal endophyte community studies they have been isolated from diverse plant organs as branches and trunks, leaves, and roots (Schulz et al. 1995). *Pezicula carpinea* (Pers.) Tul. & C. Tul. ex Fuckel, type species of the genus *Pezicula*, produces stromatal masses that break through layers of recently died bark of the host *Carpinus betulus*, on which eustromatic conidiomata of the Cryptosporiopsis asexual state develop, often also accompanied by clusters of the brightly colored, pruinose apothecia that produce eight ellipsoid aseptate ascospores in stalked inoperculate asci (Verkley 1999). *Pezicula cinnamomea* (DC.) Sacc. has been reported to cause serious bark diseases of *Quercus rubra* (Kehr 1991,1992). Notorious pathogens are also found in the closely related genus *Neofabraea* H. S. Jacks. (previously regarded as a synonym of *Pezicula* by taxonomists) (Abeln et al. 2000; de Jong et al. 2001).

* *Corresponding author*. Tel.: +86 (571) 63105091; fax: +86 (571) 63341304.
E-mail address: zlyuanmycology@gmail.com (Z. Yuan).
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Verkley (1999) monographed *Pezicula* and could largely resolve the till then confusing taxonomy of the common species *P. cinnamomea* (including *P. livida* (Berk. & Broome) Rehm) and *P. eucrita* (P. Karst.) P. Karst. He introduced a new species, *P. sporulosa* Verkley, for material that was morphologically distinct from both species, and also differed in PCR-based RFLP patterns of rRNA genes. Some material on *Abies alba* that was morphologically indistinguishable from *P. sporulosa*, showed differences in RFLP patterns but it remained unclear at that time whether it should be regarded as specifically distinct or conspecific with *P. sporulosa*. In the present study this material and its relatives were again included and, based on multi-locus sequence data, found to be conspecific with an undescribed, common endophytic species of *A. beshanzuensis* in China.

2. Materials and methods

2.1. Strains used in this study

Microscopic examination of fresh and rehydrated material of the type specimen of the new species described in this paper, and culture studies of ex-type strains were performed as described by Verkley (1999). Sporulating structures in vitro described in this study were obtained from oatmeal (OA) and malt extract agar (MEA) plates after 14–21 d of incubation at 18 °C under near-UV light (12 h light:12 h dark).

The endophytic *Pezicula* isolates were recovered from conifer tissues in *Abies beshanzuensis* (Zhejiang province, P.R. China) as described by Yuan et al. (2011). As a second dominant colonizer, more than 200 isolates were obtained. Of them, 33 strains with highly divergent phenotypes were selected for molecular analysis in this study. Unfortunately, all isolates did not sporulate in vitro.

2.2. Fungal DNA extraction and PCR amplification of four gene markers

Totally, genomic DNA was extracted from 25 *Pezicula* strains (11 species) preserved in CBS-KNAW Biodiversity Centre (Utrecht, the Netherlands), and 33 endophytic isolates (Table 1). Fresh mycelia were scraped off from cultures growing on PDA medium. The Multisource Genomic DNA Miniprep Kit (Axygen Inc., Hangzhou, China) was used to extract fungal genomic DNA following the manufacturer's instructions.

The 5.8S gene and flanking internal transcribed spacer (ITS1 and ITS2) regions of rRNA gene from all isolates were amplified using the fungal specific primer set (ITS1F and ITS4) (White et al. 1990; Gardes and Bruns 1993), and the partial RPB2 gene (RNA polymerase II second largest subunit) by using the primer set (RPB2-5f and RPB2-7cr) (Liu et al. 1999). The TEF-1α product 983F-2218R was amplified with the thermo-cycler protocol by Rehner and Buckley (2005) and the β-tubulin gene by using the forward primer Bt-T2m-Up and reverse primer Bt-LEV-Lo1 (de Jong et al. 2001). In cases where the Bt-T2m-Up and Bt-LEV-Lo1 primers did not work well, T1-T22 primer pair was used (O'Donnell and Cigelnik 1997) for amplification. All PCR products were sequenced with primers ITS1F and ITS4 for (ITS); RPB2-5f and RPB2-7cr for (RPB2); 983F, 1577F, 2218R (Rehner and Buckley 2005) for (TEF-1α); T1, T12, T22 for β-tubulin. The manually edited sequences of these four genetic loci were deposited at GenBank and the accession numbers were listed in Table 1.

2.3. Phylogenetic analyses

For TEF-1α and β-tubulin, the introns were removed from sequences according to published data. Raw DNA sequences were then subject to a multiple sequence alignment in MEGA (version 5.2) using Muscle algorithms. Each single gene alignment was analyzed individually. Ambiguously aligned regions were excluded from the phylogenetic analyses. The alignment was finally manually modified using GENEDOC (Nicholas and Nicholas 1997) when necessary. Alignments were exported as NEXUS format and maximum parsimony analysis was performed in PAUP* 4.0b10 (Swofford 2003), parsimonious trees were found through a heuristic search strategy using tree bisection-reconnection (TBR) branch swapping. Stability of clades was tested using 1000 bootstrap replications.

The four individually aligned gene regions were concatenated into a single file. The partition homogeneity test (incongruence length difference test) was used to test for conflict among the genes as implemented in PAUP with 500 replicates. A maximum likelihood search was performed using the RAxML BlackBox (http://embnet.vital-it.ch/raxml-bb/) with gamma partitioned model (Stamatakis et al. 2008). Node support was assessed with 100 rapid bootstrap replicates using RAxML. To determine the model of nucleotide substitution that best fits the sequence dataset, jModelTest version 2.1.3 (Darriba et al. 2012) was conducted using AIC criterion. Several GTR models without parameters were used as the priors in MrBayes. The priors for each datasets were: (i) ITS = GTR + I; (ii) TEF-1α = TrNef + G; (iii) RPB2 = TrN + I + G; (iv) β-tubulin = TIM2+I; (v) the combined gene was unlinked in Statefreq, Revmat and Pinvar so that each partition (gene) could be applied with the corresponding GTR model described above. Phylogenetic trees were inferred with the Markov chain Monte Carlo (MCMC) algorithm as implemented in MrBayes version 3.1.2 software (Ronquist and Huelsenbeck 2003). Convergence of the two runs, evaluated by a standard deviation of split frequencies <0.01, was reached after at least 1–2 million generations. Two independent runs were started simultaneously, with four chains in each run (one heat chain and three cold chains) heated by a temperature of 0.3. The first 25% sampled trees were discarded as burnin. All trees were rooted by two outgroups, *Cryptosporiopsis actinidiae* P. R. Johnst., M. A. Manning & X. Meier and an unidentified Dermateaceae sp. Bootstrap values (≥0.65) by either of the maximum likelihood (ML) or the maximum parsimony (MP) methods and posterior probabilities (≥0.90) of Bayesian inference (PP) are shown on the nodes (see Fig. 3).

3. Results

3.1. Taxonomy

Pezicula neosporulosa Z.L. Yuan & Verkley, sp. nov.

Figs. 1, 2.