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Molecular characterization of strawberry pathogen *Gnomonia fragariae* and its genetic relatedness to other *Gnomonia* species and members of *Diaporthales*

Inga MOROČKO[a,b,*], *Jamshid FATEHI*[a]

[a]*MASE Laboratories AB, Box 148, S-751 04, Uppsala, Sweden*
[b]*Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Box 7026, S-750 07, Uppsala, Sweden*

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ABSTRACT

Gnomonia fragariae is a poorly studied ascomycete belonging to *Diaporthales*. Originally *G. fragariae* was considered a saprophyte occurring on dead tissues of strawberry plants. Recently this fungus was found in Latvia and Sweden, and it was proven to be the cause of severe root rot and petiole blight of strawberry. Thirteen isolates of this pathogen and several other *Gnomonia* species occurring on rosaceous hosts were characterized by molecular analysis using nucleotide sequences of partial LSU rRNA gene and the total ITS region. The homologous regions from relevant diaporthalean taxa available in the GenBank were also included and compared with the taxa sequenced in this study. Phylogenetic analyses revealed that *G. fragariae*, *G. rubi*, and *Gnomonia* sp. (CBS 850.79) were genetically different from *G. gnomon*, the type species of the genus, and other members of *Gnomoniaceae*. The analyses showed that *G. fragariae* and *Hapalocystis* were genetically very closely related, forming a phylogenetic clade, which is possibly presenting a new family in the *Diaporthales*. Morphological comparisons of the *Gnomonia* species on the basis of commonly used criteria for the taxonomy of *Diaporthales*, so far did not reveal any evident features for the polyphyletic status of *Gnomonia*.

Introduction

Gnomonia fragariae is a poorly studied ascomycete in the order *Diaporthales*, with apparently limited distribution in Europe and with no known anamorphic state. In nature, *G. fragariae* has been found on dead tissues of various species of *Potentilla* and *Fragaria*, including cultivated strawberries (Bolay 1972). Originally, *G. fragariae* was found and described on petioles of dead leaves, during the screening for causes of diseased strawberries in Germany (Klebahn 1918). Klebahn considered the fungus as a saprophyte living on dead tissues of strawberry plants because he could not ascertain its pathogenicity. Recently, *G. fragariae* was repeatedly isolated during surveys for possible fungal causes of severe strawberry decline observed in several fields in Latvia and Sweden, and it was proven to be a serious pathogen involved in root rot complex and cause of root rot and petiole blight of strawberry (Morocko *et al.* 2006). Another species of *Gnomonia*, *G. comari*, is a worldwide pathogen of strawberry, and is mostly known in its anamorphic state, *Zythia fragariae*. This fungus has been reported to be a cause of strawberry leaf blotch, fruit rot, and stem-end rot (Alexopoulos & Cation 1948; Shipton 1967; Bolay 1972; Gubler & Feliciano 1999). However, *G. comari* is considered to be a weak pathogen that rarely causes considerable losses

* Corresponding author. Latvia State Institute of Fruit-Growing, Graudu str. 1, LV-3701, Dobele, Latvia. Tel.: +371 372 2294
E-mail address: inga.morocko@lvai.lv
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(Bolay 1972). It has also been shown to induce petiole blight (van Adriechem & Bosher 1958) and root rot, particularly in a synergistic interaction with nematodes (Kurppa & Vrain 1989). In our previous study, *G. comari* was seldom found on petioles of strawberry plants together with *G. fragariae* (Morocko *et al.* 2006).

The order *Diaporthales*, to which *Gnomonia* belongs, is characterized by the presence of brown to black perithecial ascomata immersed in substrate or stroma, lack of true paraphyses at maturity, and unitunicate asci with refractive ring at the apex (Barr 1978; Castlebury *et al.* 2002). *Diaporthales* includes a number of plant pathogenic fungi mostly considered as facultative pathogens, and a few systemic pathogens causing cankers and dieback symptoms. The latter include *Cryphonectria parasitica* (chestnut blight), *Diaporthe phaseolorum* (soybean stem canker) and *D. citri* (stem-end rot of citrus) are known as the most destructive (Farr *et al.* 2001; Castlebury *et al.* 2002). *Gnomoniaceae*, one of the major families in *Diaporthales*, is characterized by solitary, upright ascomata with usually central beaks and immersed in substrate or aggregated in reduced stromata, having non-septate, one-septate or occasionally several septate ascospores (Barr 1978; Monod 1983). A recent molecular study on phylogenetic relationships among 82 diaporthalean taxa have principally supported the existing concept of *Gnomoniaceae* and several other families within *Diaporthales* (Castlebury *et al.* 2002).

According to Monod (1983), the genus *Gnomonia* is characterized by ascomata immersed in, or liberated from, host tissues at maturity, containing asci with two, four, and most often eight or 20–30 hyaline, straight, or slightly curved ascospores. These spores are one septate at the middle or between middle and two-fifths of the length with little or no constriction at the septum, often with thread-like or spathuliform appendages at both ends. *Gnomonia* species are common, but not well studied, and there has been much confusion regarding the type species of the genus. Two species, *G. gnomon* and *G. setacea*, in the literature had been assigned as the type species, and confusion had existed on the identities of these two species names. However, earlier Monod (1983) separated these two taxa on the basis of morphology, and recently Sogonov *et al.* (2005) compared these species using morphological and molecular characters, and it was also shown that *G. setacea* was distinct from *G. gnomon*, the type species of the genus. Most species of *Gnomonia* are saprophytic, but the genus also includes several plant pathogens, which attack aerial constituents of herbaceous and woody hosts (Barr 1978). Several species of this genus are associated with rosaceous plants; among them *G. rubi* and *G. rostellata* are causal agents of severe cankers and dieback of different cultivated and wild *Rubus* species and roses both in Europe and America (Schneider *et al.* 1969; Ellis *et al.* 1984; Maas *et al.* 1989; Arsenijević & Veselić 1995; Nordskog *et al.* 2003). Based on morphological characters some authors have considered these two species as synonyms (Monod 1983; Barr 1991). Apart from *G. fragariae*, the only other species of *Gnomonia* known as a root-infecting pathogen is *G. radicicola*, which has been described as a cause of severe root rot of roses in artificial substrates in greenhouses (Noordeloos *et al.* 1989; Amsing 1995).

The nucleotide sequences of the rRNA genes have been widely used to distinguish taxa at both species and genus level, and also to infer phylogeny of different taxonomic ranks in fungi (Bruns *et al.* 1991; Bruns *et al.* 1992; Moncalvo *et al.* 2002). The aim of present study was to characterize *G. fragariae* isolates originating from strawberry, by using nucleotide sequences of the complete ITS, including ITS1 and ITS2, 5.8S rDNA, and a segment of the LSU rRNA gene, and to infer genetic relationships of theses isolates with other *Gnomonia* species and members of *Diaporthales*.

Materials and methods

Isolates, culture maintenance, and morphological examination

Nineteen isolates belonging to six species were used in this study (Table 1). *Gnomonia fragariae* and *G. comari* isolates obtained in our previous study (Morocko *et al.* 2006) are maintained in active state on potato-dextrose agar (PDA; Oxoid, Basingstoke) and oatmeal agar (OA; Difco, Detroit) slants at 4 °C, at the Latvia State Institute of Fruit-Growing, Latvia.

For morphological examination, the isolates were grown on OA, potato–carrot agar (PCA) and water agar (Difco, Detroit) plates to which autoclaved toothpicks or pieces of leaves and stems of *Fragaria*, *Rubus* or *Rosae* (host plants from which fungi were originally isolated), were added. Plates were incubated at 19 °C with 12 h daily illumination of black, and a light-blue (Sylwania F15W/BLB-T8) and cool-white (Osram L 15W/840) lamp combination.

DNA extraction, PCR amplification, and sequencing

To obtain mycelium for DNA extraction, 20 ml potato–carrot broth (PCB; Dhingra & Sinclair 1995) in Petri plates were inoculated with several mycelial plugs (5 mm diam) taken from 7–11 d-old fungal colonies grown on PDA or PCA. Plates were incubated at room temperature for 8–13 d in stationary conditions. To harvest mycelia, liquid medium was decanted and agar plugs were removed. Mycelial mats were rinsed three times with sterile distilled water and blot dried with sterile filter paper. Harvested mycelia were freeze-dried before DNA extraction. DNA was extracted with DNeasy Plant Mini kit (Qiagen, Crawley) according to the manufacturer's instructions.

The amplification of nuLSU rDNA was performed with puReTaq Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Piscataway) with primers LR0R and LR5 (Vilgalys & Hester 1990) following the manufacturer's instructions. The amplification was carried out in Perkin Elmer PCR thermocycler, at one cycle of 95 °C for 5 min, and 35 cycles consisting of 1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C followed by a 15 min extension step at 72 °C. The total ITS regions and 5.8S subunit of the rRNA gene cluster was amplified with primer pair ITS1 and ITS4 or ITS 1-F and ITS4 (Gardes & Bruns 1993; White *et al.* 1990) using puReTaq Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Piscataway) or in 50 μl reaction containing 3 μl genomic DNA, 5 μl of 10 μM each dNTP, 3 μl of 10 μM each primer, 2 μl dimethyl sulphoxide (DMSO; Sigma-Aldrich, Deissenhofen), 3 μl of 25 mM $MgCl_2$, 1.5 units of REDTaq (Sigma-Aldrich, Deissenhofen) and 5 μl of supplied