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SHORT COMMUNICATION

Morphological and Molecular Characterization of *Phoma complanata*, a New Causal Agent of *Archangelica officinalis* Hoffm. in Poland

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Abstract

The paper concerns the fungus *Phoma complanata*, isolated for the first time in Poland, from the roots and umbels of angelica (*Archangelica officinalis*) in 2009. The morphology of fungal isolates was tested on standard culture media. Moreover, the sequence analysis of ITS regions was conducted. Morphological similarity of *P. complanata* Polish isolates to the reference isolate obtained from CBS culture collection was determined and together with the molecular analysis confirmed the affiliation of the fungus to the species.

Key words: *Phoma complanata*, *fungus Phoma sensu lato from* angelica, ITS rDNA sequences, SEM identification

Phoma sensu lato is a highly polyphyletic genus with its unclear species boundaries (Aveskamp *et al*., 2008; 2010; Rai *et al.,* 2014; Chen *et al.*, 2015). The conventional system of identification based on morphological features in *in vitro* conditions is still valid but insufficient. Increasingly, in order to achieve the correct identification of *Phoma sensu lato*, secondary metabolites, the protein profile and nucleotide sequences using modern molecular techniques have been examined (Aveskamp *et al*., 2008; 2010; Frisvad *et al*., 2008; Rai *et al*., 2014).

P. complanata according to the current rules of taxonomy, belongs to the family *Didymellaceae*, which according to the old system included species of the section *Phoma*, *Peyronella*, *Heterospora* oraz *ParaPhoma* (Aveskamp *et al*., 2010).

Farr *et al*. (1995) reported occurrence of *P. complanata* isolates on angelica stem in the USA. On the other hand, according to Boerema *et al*. (2004) the species *P. complanata* is commonly transferred by the seeds of parsnip (*Pastinaca sativa)*, parsley (*Petroselinm crispum*) and carrots (*Daucus carota*), and damaged petioles, leaves and roots of these plants.

P. complanata was isolated for the first time in Poland from the roots and umbels of angelica (*Archangelica officinalis*) in 2009 (Zalewska *et al*., 2013). The isolation of *P. complanata* was repeated in recent years.

The accessible literature provides information on disease symptoms caused by *P. complanata* (Farr *et al*., 1995; Zalewska *et al*., 2013), pathogenicity and the mode of penetration of angelica leave and stem tissue (oral communication). The present research undertakes identification with morphological features Polish isolates of *P. complanata*. Moreover, the sequence analysis of the ITS regions was carried out – in order to confirm the accuracy of identification.

In the studies there were used single-cultures of *P. complanata* (Tode) Desm. from the collection of the Department of Phytopathology and Mycology of the University of Life Sciences in Lublin. These cultures were obtained from angelica leaves (Zalewska *et al*., 2013) and identified on standard media basing on a study Boerema *et al*. (2004), taking into account the up to date rules of taxonomy of *Phoma* genus, while reference isolate CBS 100311 was from the stems of hogweed (*Heracleum spondyllium* L.) in the Netherlands obtained from Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Three randomly selected isolates of *P. complanata*: A 103, A 233 and A 235 and reference isolate CBS 100311 were the subject of morphological and genetic characteristics.

The 3 mm discs of sporulating mycelium of the above mentioned isolates were placed on three solidified standard media, *i.e.* MA – maltose agar medium, OA – oat agar medium and CA – cherry agar medium (Boerema *et al*., 2004). The mode of the culture incubation and description is provided in Boerema *et al*. (2004). The measurements of 300 conidia (3 isolates × 100 conidia)

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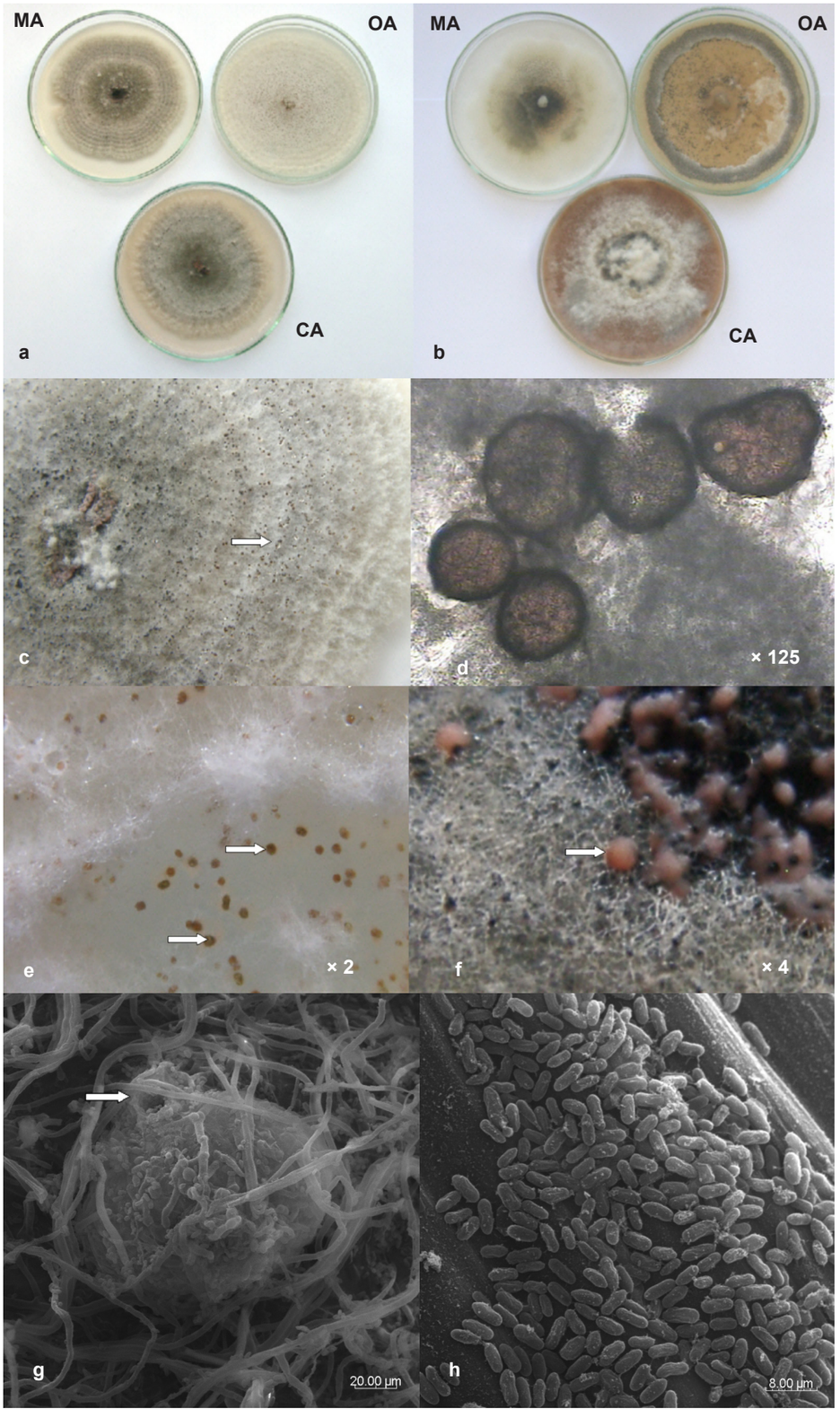

Fig. 1. *P. complanata* morphology.
(a) 7-day-old colonies on standard media. (b) 14 –day-old colonies on standard media. (c) pycnidia (arrow) in the aerial mycelium. (d) aggregate of pycnidia (×125). (e, f) drops of conidial exudate on OA (arrows). (g) Scanning electron micrograph of pycnidium with ostiole (arrow) (scale bar = 20.00 µm). (h) Scanning electron micrograph of conidia (scale bar = 8.00 µm).

and 150 pycnidia (3 isolates × 50 pycnidia) were performed after 2 weeks of culture on the oat agar medium (OA). The presence of chlamydospores was also detected. Documentation was made using the light and scanning electron microscopy (SEM).

Genetic identification was based on the differences in the nucleotide sequences of the PCR-amplified fragments of ITS regions of rDNA (ITS1, 5.8S r DNA gene, ITS2). ITS fragments were amplified with two sets of primers ITS1 and ITS4 (White *et al*., 1990).

Sequencing of the PCR products was made by the company Genomed S.A. Poland. The obtained nucleotide sequences were analysed with clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) software and compared with sequences collected in NCBI Gene Bank databases with Blast software (http://www.ncbi.nlm.nih.gov.BLAST/). The phylogenetic analysis were performed using the Phylogeny. fr program (http://www.phylogeny.fr/simple_phylogeny.cgi). The original DNA sequences obtained in this study have been deposited in GenBank.

Morphological studies showed that the growth of *P. complanata* isolates on MA was zoned. The colonies were cream-olive to grey floccose, aerial mycelium with a regular edge and a clear margin (Fig. 1a). The reverse of the colony was olive (Table I). After 14 days the mycelium formed a compact floccose to woolly structure more than after 7 days (Fig. 1b) . The diameter of the colony after 7 and 14 days was, 35–37 and 73–86 mm respectively (Table I). Colonies on OA after 7 and 14 days were white-gray with a bright-olive reverse and floccose to woolly aerial mycelium. The edge of the colonies was regular (Fig. 1a, b). The diameter of the colony after 7 and 14 days on OA was, 36 and 82–84 mm respectively (Table I). Colonies on CA after 7 days were gray-olive, dark in the oldest part of the colony, with a bright 1 cm margin. The reverse was dark-olive. The aerial mycelium was at the beginning floccose, but after 14 days it gradually became more compact and woolly (Fig. 1b). The diameter of the colony after 7 and 14 days on CA was, 34–36 and 76 mm respectively. The edge was regular (Table I). Application of a droplet of NaOH after 14 days did not have any effect. The crystals didn't form. The pycnidia were formed on all media after 7 days in the oldest part of the colonies, singly or in small aggregates (Fig. 1c, d) and secreted beige to rose exudate of conidia (Fig. 1e, f). The pycnidial walls were multilayer, thick, with one ostiole (Fig. 1g). The size of the pycnidia ranged from 86 to 288 µm (Table II). The conidia were differentiated in shape and size, usually oval, cylindrical, ellipsoidal, mostly aseptate, and 2.86 – 7.64 × 1.91 – 3.82 µm in dimension (Table II, Fig. 1h). Occasionally, 1-septate conidia with the dimension of 9.55–13.37 × 2.86–3.83 µm were observed in 14-old-days cultures. Similarly, in the case of isolate CBS 100311 1-septate conidia with the dimension of 14.21–18.23 × 4.33–6.11 µm constituted about 2% on 14-day-old cultures grown on OA medium (Table II).

Electrophoresis of PCR amplification products revealed a distinct band of approximately 550 bp. Nucleotide sequences of the ITS region from A 103, A 233 and A 235 of *P. complanata* isolates were identical. However, ITS sequences from these isolates slightly differed from the reference isolate by some substitutions and alignment gaps within ITS region. The amplified fragment showed 96% identity on the length of 434 bp for isolate A 103, 432 bp for isolate A 233 and 433 bp for isolate A 235 with nucleotide sequence of *P. complanata* collected in the CBS. A phylogenetic tree, based on the ITS sequence of three isolates of *P. complanata* and reference strain generated using the Phylogeny. fr analysis, indicated the segregation of all isolates into two main clusters. The first cluster grouped reference strain and our three native isolates of *P. complanata*: A 103, A 233 and A 235. The second cluster included *P. neerlandica* CBS 134.96 , the isolate which has been used the tree to be rooted (Fig. 2). Sequences of above isolates have been deposited in GenBank, respectively with the reference numbers MF062524, MF062525 and MF062524. Sequence-based identification was correlated with the identification by classical methods.

Genus *Phoma* discussed by Boerema *et al.* in 2004 and described in the 10th Edition, Dictionary of the Fungi "(Kirk *et al*., 2008), now should be considered as *sensu lato* because it involves a group of about 10 different genera, four already known and some new (Aveskamp *et al*., 2010; De Gruyter, 2012). The current taxonomical system based on phylogenetic analysis abolished the previous division into sections and made it necessary to reclassify *Phoma* (Aveskamp *et al.,* 2010; De Gruyter, 2012). Research conducted by Dutch scientists led to a division of genus of *Phoma sensu lato* into clades and groups that include species with a similar degree of relationship. Some of them are now raised to the level of genus.

P. complanata was classified as *Didymellaceae* family, which included the species of *Phoma* that previously belonged to the sections *Phoma*, *Phyllostictioides*, *Peyronellaea*, *Sclerophomella*, *Macrospora* and some phytopathologically similar species from the sections *Heterospora* and *ParaPhoma* are found in *Didymellaceae*

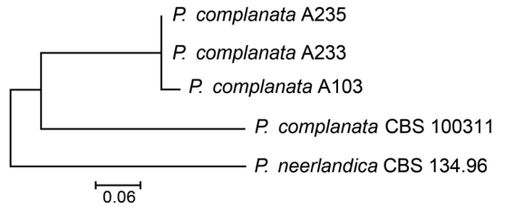

Fig. 2. Phylogenetic tree of native isolates of *P. complanata* and reference strain generated from Phylogeny. fr analysis of the ITS.

Table I
Features of *P. complanata* cultures on standard medium (mean for 3 isolates)

Medium	Malt Agar Medium (MA)		Oatmeal Agar medium (OA)		Cherry Agar Medium (CA)		According to Boerema *et al.* (2004)		
The studied features	After 7 days	After 14 days	After 7 days	After 14 days	After 7 days	After 14 days	MA	OA	CA
Diameter of colonies	35–37 mm	73–86 mm	36 mm	82–84 mm	34–36 mm	76 mm	59–79 mm	60 – 82 mm	49–79
Colour of averse	cream-olivaceous	cream-olivaceous	white-grey	white-grey	grey--olivaceous	grey olivaceous	colourless to primrose with citrine green to olivaceous tingers	colourless or buff to greenish olivaceous	colourless/saffron greenish olivaceous or olivaceous
Colour of reverse	olivaceous	pale-olivaceous	pale-olivaceous	pale-olivaceous	olivaceous	dark olivaceous	colourless to primrose with citrine green to olivaceous tingers	primrose to salmon or citrine green to olivaceous in centre	saffron/fulvous to olivaceous
Character of the growth of colonies margin	regular	regular	regular	regular	regular	regular	regular	regular	regular or slighty irregular
Structure of aerial mycelium	floccose	floccose to woolly compact	floccose	floccose to woolly	floccose	floccose to wolly compact	velvety to floccose woolly, compact	floccose to woolly, sometimes compact	woolly to floccose
Colour of cultures after reaction with 1N NaOH		negative		negative			negative	negative	

Table II
Features of pycnidia and conidia of *P. complanata* on oat medium (mean for 3 isolates)

Author	*Phoma complanata*		
	Pycnidia		Conidia
	a	b	a
Own data	globose to irregular without visible ostiole 86–288 µm	on the agar, partly submerged in the aerial hyphae of mycelium, olivaceous black with buff exudate of conidia	subglobose, cylindrical, ellipsoidal, mostly aseptate 2.86–7.64 × 1.91–3.82 µm and 1-septate conidia 9.55–13.37 × 2.86–3.82 µm with small guttules
References strain CBS 100311	globose to irregular, without visible ostiole, mostly 85–252 µm	glabrous, olivaceous-black with buff to salmon exudate of conidia, on the agar or partly submerged in the agar, solitary or sometimes aggregated	subglobos, ellipsoidal, cylindrical to fusiform, mostly aseptate 3.85–10.53 × 2.28–4.33 µm, 1-septate conidia 14.21–18.23 × 4.33 – 6.11 µm with small guttules
Boerema *et al.* 2004	globose to irregular, mostly 80–240 µm, with 1 non-papillate pore	glabrous, finally olivaceous black, solitary or confluent with buff to rosy exudate of conidia, walls made up of 2–6 layers of cells, outer layers pigmental	variable in shape and size, subglobose, ellipsoidal, cylindrical to fusiform, mostly aseptate 3–11 × 1.5–4 µm, usually 5–10 × 2–3 µm, sometimes in fresh culture 1-septate up to 16 × 4 µm, in oloder cultures large 22–34 × 6–10 µm, usually with several small guttules

a – shape and dimension in µm
b – arrangement and structure of wall surface

(Aveskamp *et al*., 2010). Despite the reclassification of species of *Phoma sensu lato*, the identification system based on constant macro-and microscopic features, physiological and biochemical characteristics observed *in vitro* in cultures developing in standard conditions is still valid (Aveskamp *et al*., 2010; De Gruyter, 2012). The study on the morphology and growth of Polish isolates of *P. complanata* was consistent with the description given by Boerema *et al*. (2004) and allowed to identify the species as *P. complanata*. In addition, the morphological and genetic similarity to the reference isolate from CBS has been proven. The Polish isolates of *P. complanata* on OA formed mainly aseptate conidia. In the case of isolate CBS 1-septate conidia were observed. Their share was about 2%. It is known from the literature that in the genus *Phoma sensu lato* the conidial septa formed secondarily, regardless of the conidiogenesis process, so a small percentage of spores may have secondary septa (Boerema and Bollen, 1975). It means that the morphological characteristics of conidia of these fungi are significant in secondary diagnostics. Literature reports the possibility of the occurrence of variation in morphological and physiological features between isolates obtained from different host plants, which may explain the absence or occasional presence of 1-septate conidia of native isolates of *P. complanata* (Koike *et al*., 2006).

Demonstrated in the present study close similarity in ITS sequence within our isolates demonstrated in this study confirms that they represent the same species of fungus. Moreover morphological characteristics and analysis of ITS1, ITS2 nucleotide sequence leads to the conclusion that isolates belong to *P. complanata* species. It seems that small differences between the three studied isolates and the reference isolate of *P. complanata* are possible, as in the case of species belonging to other taxa (Uddin *et al*., 1998). However, according to some authors ITS sequence did not provide unambiguous identification and additional sequencing of other gene fragments is required (Balmas *et al*., 2005; Woudenberg *et al*., 2009; Błaszczak *et al*., 2011). And so, the current results suggested that further research is needed to differentiation within of *P. complanata* isolates.

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