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APPLICATION OF POLYPHASIC APPROACH FOR IDENTIFICATION OF EPIPHYTIC FUNGI ISOLATED FROM WHEAT GRAINS

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Abstract

In this study, epiphytic fungi were isolated from wheat seeds (*Triticum aestivum* L) and a polyphasic approach for their identification was applied. The initial morphological characterisation was done on selected twenty-tree strains and continued with their identification based on the carbon utilisation pattern according to the Biolog OmniLog system protocol (Hayward, CA, USA). Throughout the isolated strains, the Biolog data indicated predominance of the genera *Aspergillus*, *Penicillium* and *Fusarium.* The carbon utilisation pattern of five strains assigned them to several different from aforementioned genera and due to their scarcity they were not included in the current study. The indicated by the Biolog genus designation of the strains was used as a guideline for the further molecular identification of seventeen strains from the predominant genera. Molecular identification was based on a polymerase chain reaction (PCR) with universal primers, which targeted the internal transcribed spacer (ITS) region of rDNA gene. The PCR fragments were sequenced and after applying a BLAST algorithm, a high percentage of similarity was found for eleven strains. At the species level, the Biolog and molecular technique showed a discrepancy in the identification of two strains. The procedure of identification was unsuccessful for six strains either due to insufficient quantity of the PCR product or the lack of sufficient similarity of the fragments to the GenBank database. Current study showed that the unbiased identification of epiphytic fungi requires a polyphasic approach, which applies morphological, physiological and molecular techniques.

Keywords: epiphytic fungi, PCR, Biolog OmniLog system, internal transcribed spacer (ITS), GenBank

INTRODUCTION

The identification of fungi has traditionally relied on combined macroscopic colony morphology and a meticulous microscopic characterisation of their mycelium after several days of cultivation at suitable temperature (Hyde et al., 2010). More detailed and precise characterisation depends on the thorough observation of the fungal reproductive bodies, type, shape, colour and numbers of conidia. In any case the correct species identification requires knowledge, training and extensive expertise from the person responsible for the fungal recognition (Diba et al., 2007, Bandh et al., 2011). However, in certain cases, specialised media is necessary to stimulate formation of fruiting bodies but it is also possible that the specific strains of the fungi would not form conidia, which would restrain their successful identification (Hinrikson et al., 2005). Furthermore, the morphological or cultural attributes such as conidiophore elongations and ornamentation, or conidial colour, which can be very efficiently used to distinguish isolates, still may not reflect the true phylogenetic relationships between the fungal strains (Kindermann et al., 1998). Along with methods for morphological characterisation a carbon source utilisation (microarrays

technology) for strains identification is also available option (Dobranic & Zak, 1999, Atanasova et al., 2010, Pinzari et al., 2016). A Biolog OmniLog system (Inc., Hayward, CA) which applies phenotype microarrays technology initially was designed for unicellular prokaryotes, but then the methodology was optimised and improved. As a result, the accuracy of fungi identification based on their carbon utilisation profile has increased (Tanzer et al., 2003). Despite the extended species record in the Biolog database, the comparison of the newly isolated fungal strains with the available profiles in the system is not always successful. The fungal identification or results from the carbon utilization pattern could be dubious or not satisfactory (Wünsche & Babel, 1996). Currently, several rapid, nucleic acid-based methods, which attempt to identify fungal genera independently of their morphology or to replace the methods for phenotypic identification, especially for clinically and environmentally important genera such as *Aspergillus*, *Penicillium* and *Fusarium*, are used (Tsui et al., 2011, Aslam et al., 2017). In several occasions, the sequence analyses have already opposed the identification derived from the morphology characterisation of some well defined at species level fungi, because clearly revealed the existence of phylogenetically different species (Hyde et al., 2010). According to Hinrikson et al. (2005), the most successful methods used polymerase chain reaction (PCR) amplification of target regions within the ribosomal DNA gene complex, including 28S ribosomal subunit (D1-D2 region) and the internal transcribed spacers 1 and 2 (ITS1 and ITS2 regions). Such DNA fragments often are denoted also as DNA barcodes and are considered as a useful "formula" for species identification (Hyde et al., 2010). After the pioneering work of White (White et al., 1990) and Bruns (Bruns et al., 1991) and some evidences that have supported the ITS regions sensitivity on species level identification (Gardes and Bruns, 1993, 1996) the ITS was proposed as a standard barcode for fungi (Chase & Fray., 2009) and was broadly used ever since (Schoch et al., 2012). More recently the *Fungal Working Group* have also agreed that the internal transcribed spacer (ITS) possess a broad utility as a species marker and can be used in taxonomic and ecological studies due to its specificity (Crous et al., 2015, Badotti et al., 2017). The usefulness of the ITS lies in the unique DNA sequence with length of 400–800 nucleotides which can be quickly processed and analysed by computer programs. However, many species of plant-pathogenic fungi (e.g., *Alternaria*, *Botryosphaeria*, *Calonectria*, *Cercospora*, *Diaporthe*, *Fusarium*, *Ilyonectria*, *Teratosphaeria*, etc.) could not be accurately identified with the ITS region and in such cases secondary barcodes related to protein-coding genes have to be employed (Crous et al., 2015). Tekpinar & Kalmer (2019) considered that several protein-coding regions such as TEF1-α, RPB1, RPB2, β-tubulin and CaM genes could be accepted as secondary barcodes for *Ascomycota* genera such as *Penicillium*, *Aspergillus* and *Fusarium*.

Currently, several databases provide a collection of sequences that can be used for fungal species level identification. The most used ones are: The National Center for Biotechnology Information (NCBI) database (Robbertse & Tatusova, 2011), the User-friendly Nordic ITS Ectomycorrhiza Database - UNITE database (Kõljalg et al., 2013, Nilsson et al., 2019), a Global fungi (Větrovský et al., 2020), the Barcode of Life Data Systems (Ratnasingham & Heber, 2007). It is highly possible that some other derivative databases such as PHYMYCO (Mahe et al., 2012) or specialised database would appear in the future along with those that are currently under development such as EPPO-Q-bank Fungi database (https://qbank.eppo.int/fungi/).

The aim of the current study was to present the application of a polyphasic approach for the identification of epiphytic fungal strains isolated from wheat seeds.

MATERIALS AND METHODS

Twenty-three strains of epiphytic fungi were isolated from undamaged wheat grains (*Triticum aestivum* L), cultivated on potato dextrose agar (PDA, HiMedia, India) and morphological and cultural characteristics of their mycelium and conidia were observed (data not shown). The phenotypic identification of strains based on their carbon utilization pattern was done by the Biolog semi-automated system (Biolog, Inc., Hayward, CA, USA). The data obtained from the Biolog system showed that eighteen strains belong to either *Fusarium*, *Aspergillus* or *Penicillium* genus (Table 1). Additionally, the system indicated a high similarity of five strains to different from the aforementioned genera as follows: ***P6*** - *Bionectria sesquicilli*, ***P20*** - *Alternaria alternata*, ***P21*** - *Curvularia lunata*, ***P22*** - *Scopulariopsis candida*, and ***P23*** - *Colletotrichum truncatum*. These strains were excluded from the analysis presented in the current study due to their scarcity in the collection. The strains ***P17*** and ***P18*** were both denoted as *Penicillium griseofulvum* but as separate records in the Biolog database and the strain ***P18*** was excluded of the molecular identification in order to avoid analysis of duplicate strains.

Table 1. Naming of the epiphytic fungal strains in the current study and their genus level designation according to the Biolog system

Strain	Biolog` genus level designation	Strain	Biolog` genus level designation
P1	*Fusarium*	***P14***	*Penicillium*
P2		***P15***	
P3		***P16***	
P4		***P17***	
P5			
P7		***P19***	
		Strains excluded from the current study	
P8	*Aspergillus*		
P9		***P6***	*Bionectria sesquicilli*
		P18	*Penicillium griseofulvum*
P10		***P20***	*Alternaria alternata*
P11		***P21***	*Curvularia lunata*
P12		***P22***	*Scopulariopsis candida*
P13		***P23***	*Colletotrichum truncatum*

In order to obtain the fungal mycelium strains were cultivated in potato-dextrose broth (PDA, Himedia, India), using 500 ml Erlenmeyer flasks (200ml broth) mounted at a rotary shaker (200 rpm), in the dark, at 25 °C for 3 d. After mechanical disruption of the mycelium by vortex mixing with glass beads, genomic DNA was extracted with a DNeasy plant minikit (Qiagen, Germany) according to the manufacturer`s instructions. The purity and concentration of the extracted DNA was estimated with a NanoDrop Lite 2000/2000c (*Thermo Fisher Scientific Inc.*). If the samples were not immediately analysed they were stored at - 20ºC.

The mix (25 µl) for the conventional PCR contained 0.25 µl AmpliTaq DNApolymerase, 4 µl GoTaq reaction buffer, 1.2 µl $MgCl_2$, 1 µl dNTPs (dATP, dCTP, dGTP, and dTTP), 1 µl of each selected primer, 5 µl of

DNA template and 11.55 µl sterile HPLC water. The PCR procedure was 40 cycles at the conditions that follows: denaturation at 92°C for 30 s, annealing at 58°C for 30 s, amplification at 70°C for 1 min. The PCR products, in quantity between 0.5 and 4 µl after mixing with a loading buffer, were separated by a gel electrophoresis (1% agarose) supplemented with ethidium bromide, at 100 V, for 30 min, in 0.5x TAE buffer.

The isolated DNA was amplified with universal primers (internal transcribed spacer region 1 - ITS1), ITS1, ITS2, ITS3, ITS4, ITS5 и LR1 according to White et al. (1990) and Van Tuinen (1998) (Table 2). To check the suitability of the chosen primers several preliminary tests were done. The results from the preliminary tests have shown that the primers ITS3 and ITS5 were not suitable because they have not generated any fragments with the extracted from the selected strains DNA (results not shown). The other primers were used in the PCR protocol along with the standard DNA ladder.

Table 2. Primers for Internal Transcribed Spacer (ITS) region of rDNA

Primer	Sequences	Reference
ITS1	TCCGTAGGTGAACCTGCGG	White et al., (1990)
ITS2	GCTGCGTTCTTCATCGATGC	
ITS3	GCATCGATGAAGAACGCAGC	
ITS4	TCCTCCGCTTATTGATATGC	
ITS5	GGAAGTAAAAGTCGTAACAAGG	
LR1	GGTTGGTTTCTTTTCCT	van Tuinen et al., (1998)

The obtained PCR fragments were further sequenced and the sequences were compared with the GenBank database using BLAST algorithm. The species designation was presented according to the percentage of similarity.

The DNA extraction, PCR products sequencing and BLAST analysis in the current study was done in collaboration with the colleagues from the Department of Phytopathology of the West Pomeranian University of Technology in Szczecin, Poland.

RESULTS AND DISCUSSION

In the current study, some preliminary experiments with primers ITS3 and ITS5 have not provided any fragments and the work continued with primers pairs ITS1/LR1, ITS1/ITS2 and ITS1/ITS4. The use of primers pair ITS1/LR1 for strain ***P1*** and ***P5*** provided PCR products with varied length between 400-600 bp. The primers pair ITS1/LR1 also provided a fragment with an approximate length of 500 bp for strain ***P7*** (Figure 1). However, for strain ***P4*** the chosen primers pair generated several different DNA fragments, including one very short (less than 400 bp) and due to the necessity of further more detailed analyses the strain ***P4*** was excluded from the ongoing protocol.

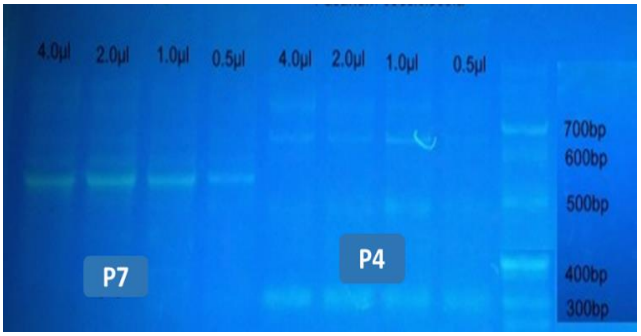

Figure 1. Agarose-gel electrophoresis of the sequences obtained for strains ***P4*** and ***P7*** with primer pairs ITS1/LR1

Strains ***P2*** and ***P3*** provided more than one clear band but among the generated fragments at least one corresponded to the approximate length of the expected PCR product (data not shown) and some additional tests with a primers pair ITS1/ITS4 were

included in the analysis. Such variability in the length of the fragments obtained with ITS primers is very usual. It was noticed by Gardes and Bruns (1993), who have detected fragments between 280 to 600 bp when ITS sequences were amplified. Similarly, when using ITS2 Chen et al. (2000) reported DNA fragments between 237 to 429 bp.

The primers pair ITS1/ITS2 was further applied for PCR method for strains assigned to the genus *Aspergillus sp*. (***P8***, ***P9***, ***P10***, ***P11*** and ***P12***) according to Sugita et al., (2004). The generated by the primers ITS1–5.8S–ITS2 fragments for strain ***P10*** were variable in length (two bands) and due to restrains in matter of time and resources was also excluded of the analysis. More successful was the analysis of strains ***P8***, ***P9***, ***P11*** and ***P12*** since each of them provided a clear band between 500-600 bp (Figure 2). The obtained results were similar to the data of Henry at al. (2000), who using primers pair ITS1/ITS4 and strains from genus *Aspergillus* sp. obtained products ranged in size from 565 to 613 bp (Henry et al., 2000). However, the comparison of the PCR fragment obtained for the strain ***P8*** have not showed a sufficient percentage of similarity with the GenBank sequence and the strain was not assigned to a species.

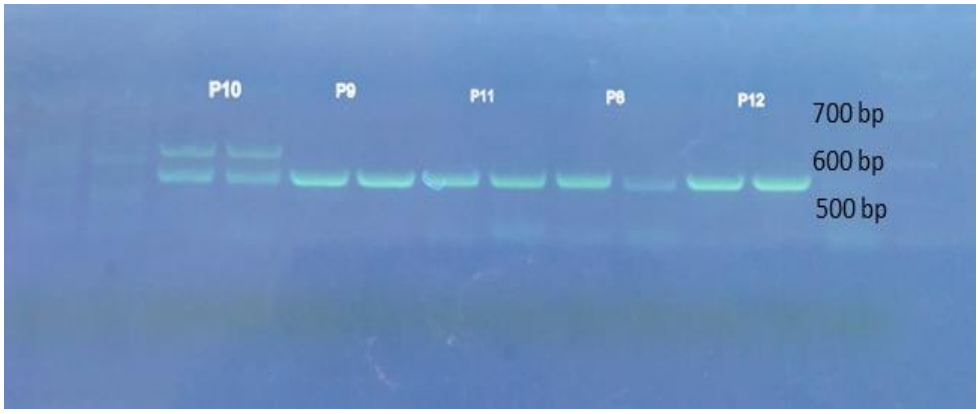

Figure 2. Agarose-gel electrophoresis of the fragments obtained for strains ***P8***, ***P9***, ***P10***, ***P11*** and ***P12*** with primers pair ITS1/ITS2

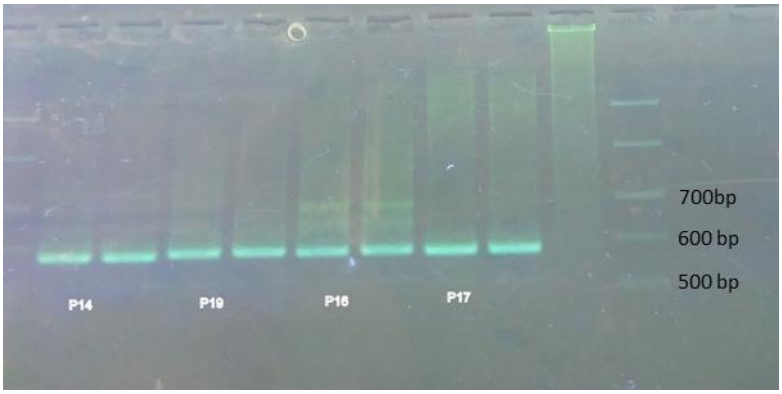

Figure 3. Agarose-gel electrophoresis of the fragments obtained for strains ***P14***, ***P16***, ***P17*** and ***P19*** with primers pair ITS1/ITS4

The usage of primers pair ITS1/ITS4 for strains ***P14***, ***P16***, ***P17*** and ***P19*** and gel electrophoresis with 1.0µl PCR products detected fragments with similar length - approximately 600 bp (Figure 3).

The preliminary experiments were not satisfactory not only for strains ***P2***, ***P3*** but also for strain ***P15*** with the application of primers

pair ITS1/LR1 and ITS1/ITS4, respectively. In order to check the primers pair effectiveness several additional tests were done with the primers pair ITS1/ITS4. The agarose-gel electrophoresis with a PCR product with quantity of 1.0µl showed products with approximate size of 400 bp from stains ***P2***, ***P3*** and ***P15*** (Figure 4). However, the comparison of the sequenced fragments showed a high percentage of similarity for strains ***P2***, ***P3*** but when the sequence for ***P15*** was compared with the GenBank it showed an unidentified at species level record (Fungal sp. 855 YZ-2011, accession number HM439537.1) and the strain ***P15*** was not assigned to a species.

The obtained PCR fragments were sequenced and compared with the GenBank database using a BLAST algorithm and the species designation was based on the percentage of similarity (table 3).

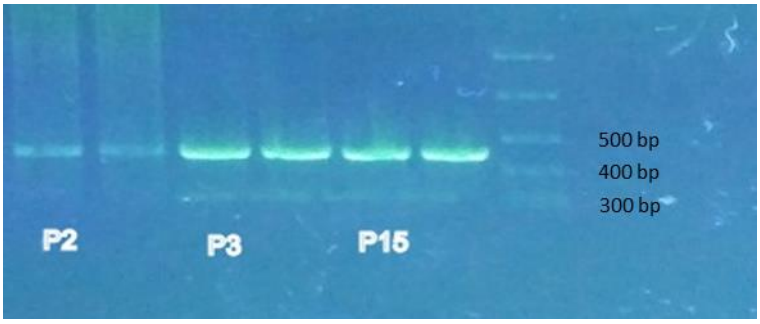

Figure 4. Agarose-gel electrophoresis of the fragments obtained for strains P2, P3 and P15 with primers pair ITS1/ITS4

Table 3. Percentage of similarity of the sequences derived with the ITS primers when compared with the GenBank database

Number in order	Strain reference	Species designation	Similarity (%)	Accession number from NCBI GenBank
1.	***P1***	*Fusarium acuminatum*	100	MH470246.1
2.	***P2***	*Fusarium equiseti*	99	MK033186.1
3.	***P3***	*Fusarium verticillioides*	100	KR047092.1
4.	***P5***	*Epicoccum nigrum*	99	MK460957.1
5.	***P7***	*Fusarium graminearum*	100	MK079920.1
6.	***P9***	*Aspergillus ochraceus*	98	EU021609.1
7.	***P11***	*Aspergillus oryzae*	100	MF663189.1
8.	***P12***	*Aspergillus ibericus*	98	EF661200.1
9.	***P14***	*Penicillium montanense*	96	HQ157959.1
10.	***P16***	*Penicillium granulatum*	99	MH393399.1
11.	***P17***	*Penicillium chrysogenum*	99	MG818936.1

A great diversity in morphology and ecology of fungi reflects on difficulties and challenges in their identification to the species level (Tekpinar & Kalmer, 2019). Along with the identification based on morphological features (Diba et al., 2007, Bandh et al., 2011) there is a significant progress in molecular identification of fungi and a broad use of PCR technique (Cuadros-Orellana et al., 2013, Crous et al., 2015). One of the most important requirements for a proper identification based on the PCR is the choice and construction of

suitable primers and conditions of the PCR. The internal transcribes spacer (ITS) primers are designed for two regions located as follows: ITS1 - 18S and 5.8S rDNA genes, and ITS2 - the 5.8S and 28S rDNA genes (Iwen et al., 2002). Bellemein et al. (2010) studied in details how the different primers pair can be applied for identification of ascomycetes and basidiomycetes. The authors used several primers pair such as ITS3-ITS4-B and ITS4 and ITS5 and have found that ITS3-ITS4-B amplified only 39.3% of the basidomycete sequences. They also reported a considerable length variation among the amplified fragments not only in the ITS1 and ITS2 regions, but also in the whole ITS region. The main problem with the primers for ITS region was that the variability in the fragments length was not consistent between the ITS regions which could be source of taxonomic bias. The fragments with different length during PCR for strains from *Aspergillus* also have been observed (Fujita et al, 2001).

Currently, the molecular techniques are an additional and valuable tool in order to obtain more knowledge about fungal genera and their species complexes. There is a lot of information about genera such as *Aspergillus* and *Penicillium*, and significant progress in collecting data related to *Fusarium* and *Colletotrichum*. It is further expected to deepened the knowledge about *Guignardia*/*Phyllosticta* and *Pestalotiopsis* (Hyde et al., 2010). However, despite the power of molecular methods, their accurateness depends on the quantity and quality of the extracted DNA and the conditions of the PCR procedure. Furthermore, the fungal identification depends on the properly chosen computer programs and algorithm for comparison, an expertise related to the available options in the existing database and the deposit of suitable sequences in them (Rajala et al., 2011). O`Brien et al. (2005) reported that while the most fungal ITS sequences could be identified, at least 12% do not provided sufficient information about the phylum identification. According to authors opinion some of these unidentifiable sequences could be novel lineages or they could represent known fungi that are going to be included in the ITS database. Despite that ITS region provides a taxonomic resolution with a high sensitivity it is considered as not sufficiently conservative for some phylogenetic analyses of unknown sequences, and there is a recommendation to apply the ITS with either SSU or LSU rRNA region (O`Brien et al., 2005).

Kwiatkowski et al (2012) have used a two-target sequencing approach for fungi identification because some of the isolates could not be amplified with the ITS universal primers even after several attempts. In order to overcome this obstacle authors proceed with the use of D1/D2 sequencing. The authors concluded that the ITS target sequencing was slightly better than D1/D2, and if there are some time and resources limitation it is worth trying to identify species only by ITS and if this analysis is unsuccessful to proceed with D1/D2. However, Hinrikson et al. (2005) identified species of *Aspergillus* by using GenBank database analysis and considered that the ITS1 and ITS2 regions were more reliable than the D1-D2 region. Bellemain et al., (2010) advised a careful selection of ITS primers along with parallel use of either different primer combinations or amplifying different parts of the ITS region. Some alternative ITS primers also could be developed and tested. Currently, the fungal taxonomic studies take into account also the intragenomic variation of rDNA. It is expected, that with advanced sequencing technologies (second and third generations), the intragenomic variation of rDNA would have a significant impact on development of DNA barcoding (Paloi et al., 2022). However, the identification with any chosen tool should be critically appraised and if it possible some additional verification procedure should take place (Lücking et al., 2020).

CONCLUSION

The proper identification of fungi is complicated and multileveled procedure which requires sufficient quantity of information related to their morphological features. The morphological characterisation that have been fundamental for the mycological taxonomy is still considered as the obligatory and necessary step in fungi identification. However, when only these characteristics are used, they very often failed to provide a reliable, conclusive and unbiased identification of fungal species. The morphological characterisation should be accompanied with more reliable and precise methods, which can be a matter of choice among the plethora of currently existing methods for molecular identification. Each of the approaches, either morphological or molecular, has its advantages and limitations but the correct identification of species belonging to such abundant and versatile domain such as fungi kingdom requires polyphasic approach for species identification. The presented study used the data based on carbon source utilization provided by the Biolog system as a source information about the genera designation of fungal strains. In the current study, the chosen primers pairs and the protocol for amplification allowed obtaining fragments, which length varied significantly between 300 and 600 bp. The identification of six strains (***P4, P8***, ***P10, P13***, ***P15, P19***) was unsuccessful either due to insufficient amplification after PCR, unsuitable fragments generated by the chosen primers, or low percentage of similarity when compared to the GenBank database. There was a discrepancy between the Biolog and the species designation based on the percentage of similarity with the sequences in the GenBank database for two strains (***P5*** and ***P17***).

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