

## ARTICLE

# Pool of endoglucanase genes in *Schizophyllum commune* Fr.:Fr. (Basidiomycetes) on the territory of Ukraine

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**ABSTRACT** Pool of intracellular endoglucanases of the fungus *Schizophyllum commune* on the territory of Ukraine was studied. Two loci of endoglucanase (*Eg1*, *Eg2*) were found. The polymorphic locus *Eg2* controls the expression of four alleles. Alleles *Eg2*<sup>93</sup>, *Eg2*<sup>96</sup> and *Eg2*<sup>102</sup> are rare and peculiar to certain populations. Amino acid sequence of the locus *Eg2* in databases of NCBI (XP\_003031634.1) and UniProt (D8Q439) was probably identified. It is classified among the family 5 (GH5) and consists of 333 amino acid residues.

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## Introduction

Wall of a plant cell is the main site where carbon sequestration takes place. In general, it consists of approximately 30% of cellulose, 30% of hemicellulose, 35% of pectin substances and 5% of structural protein. These values vary depending on the type of plant and developmental stage (Albersheim et al. 2011; Hoch 2007; Ochoa-Villarreal et al. 2012). Saprotrophic fungi use this pool of substances as an infinite source of energy for vital activities. Wood-decay fungi cause two main types of wood degradation: white and brown. White rot fungi cause degradation of all wood components including lignin (Baldrian and Valaskova 2008), while brown rot fungi cause complete degradation of polysaccharides of the cell wall and partially of lignin (Worrall et al. 1997; Yelle et al. 2008).

All processes of destruction of the plant cell wall take place with involvement of various fungal enzyme systems, therefore, these are highly favored targets of mycological (Floudas et al. 2012, 2015; Rytioja et al. 2014) and phytopathological research (Przybył et al. 2006; Simard et al. 2013). Certain fungi cause a transition type of decay (Redhead and Ginns 1985). This group includes *Schizophyllum commune* Fr.:Fr. which has a deficit in peroxidases and laccases (just like brown rot agents) and at the same time has a substantial number of enzyme systems geared to degradation of polysaccharides of a cell

wall and crystalline cellulose, just like white rot fungi do (Ohm et al. 2010). Some researchers regarded *S. commune* as a saprophyte, but others recognized the fungus as a plant pathogen that invades living tissues from wounds and potentially causes rot (Latham 1970; Takemoto et al. 2010). The fungus is identified as a pathogen in many deciduous and coniferous tree species. Due to its well-known biology and ubiquitous character, *S. commune* is a frequently studied mycological model organism (Raper 1988; Tsujijama and Ueno 2011).

One of the main enzymes taking part in the degradation of cellulose is endoglucanase (EC 3.2.1.6), that splits the inner  $\beta$ -1,4-glycosidic bonds (Schmidt and Liese 1980; Kirk and Cullen 1998; Przybył et al. 2006). Literature data show that most of the wood decaying basidiomycetes have several forms of endoglucanases, which makes this enzyme system promising for population genetic research (Baldrian and Valaskova 2008; Rytioja et al. 2014). Genome data of the *S. commune* are available that allow assessing its potentials (Ohm et al. 2010). Currently, important investigations include the research of heterogeneity of enzyme systems and their actual use (Chong et al. 2011; Song et al. 2013; Tovar-Herrera et al. 2015; Lee et al. 2014). Considering the high potential of enzyme systems of *S. commune* in the process of wood degradation and the lack of information regarding their polymorphism, our aim was to investigate the special aspects of endoglucanase pool in *S. commune* on the territory of Ukraine.

## Materials and Methods

### Organisms and culture conditions

Dikaryotic and monokaryotic cultures of *S. commune* were investigated.

Dikaryotic cultures were obtained from the basidiocarp of fungi collected in different regions of Ukraine (Chernihiv, Crimea, Donets'k, Ivano-Frankivs'k, Kyiv, Vinnytsia, and Zaporizhia region). The isolation of pure cultures from basidiocarps was performed in aseptic conditions under the MBS-10 stereomicroscope by deriving a sample of 1 × 1 mm size from the middle (sterile) part of plectenchyma and subsequently transferring it to the agar containing nutrient medium. After appearing of pure mycelium, it was repeatedly replanted to sterile nutrient.

Monokaryotic cultures were obtained using the spore print method. Water suspension of basidiospores, after multiple dilution procedures were deep plated in Petri dishes on agar medium. Purity and monosporous character of the cultures was controlled by means of microscopy. Monokaryotic cultures were used only to determine the genetic control of the locus. The total number of dikaryotic cultures was 128 (Chernihiv - 10, Crimea - 11, Donets'k - 32, Ivano-Frankivs'k - 12, Kyiv - 43, Vinnytsia - 8, and Zaporizhia - 12), while the number of monokaryotic ones was more than 500. Strains were cultivated in liquid glucose-peptone medium (10 g glucose L<sup>-1</sup>, 3 g peptone L<sup>-1</sup>, 0.4 g K<sub>2</sub>HPO<sub>4</sub> L<sup>-1</sup>, 0.5 g MgSO<sub>4</sub> × 7 H<sub>2</sub>O L<sup>-1</sup>, 0.001 g ZnSO<sub>4</sub> × 7 H<sub>2</sub>O L<sup>-1</sup>, 0.05 g CaCl<sub>2</sub> L<sup>-1</sup>) in a TC-80M thermostat at 28 °C for 15-18 days (Boiko 2011). The initial pH of the medium was 5.0.

### Histochemical detection of enzyme activity

Fungal mycelium was flushed out and dried out with the use of vacuum filtration. Subsequently, it was homogenized in Tris-citrate buffer and filtrated. Protein concentration was measured with a ULAB S131UV spectrophotometer (Layne 1957). Electrophoretic separation of proteins was performed in 11.25% polyacrylamide gel (PAAG) with the use of Tris-glycine buffer (pH 8.3). Amount of added protein in each well varied within 40-60 µg.

Detection of activity zones of endoglucanase (EG) in PAAG was performed in the presence of Na-carboxymethylcellulose (C5678, Sigma) (Manchenko 2003). After electrophoresis, the gel was washed in 50 µM phosphate buffer (pH 5.8) for 10 min and incubated at 50 °C for 60 min. Then the gel was rinsed in distilled water and placed in 0.1% Congo Red solution for 10 min at room temperature. Finally, the gel was washed in 1 M NaCl solution for 10 min. Enzyme activity resulted in light yellow bands on the red background of the gel. Electrophoretograms were assessed with the TotalLab TL 120 software.

### Statistical analysis

Genetic control of the identified electrophoretic variants of enzymes was studied by analyzing their segregation among monokaryotic cultures. Genetic diversity was characterized using allelic frequency ( $A_E$ ), Shannon's diversity index (I), expected and observed heterozygosity ( $H_o$  and  $H_e$ ), and Wright's fixation index (Nei 1978). Genetic data were calculated using the POPGENE32 software (Yeh et al. 1999). BLAST searches were conducted on database of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

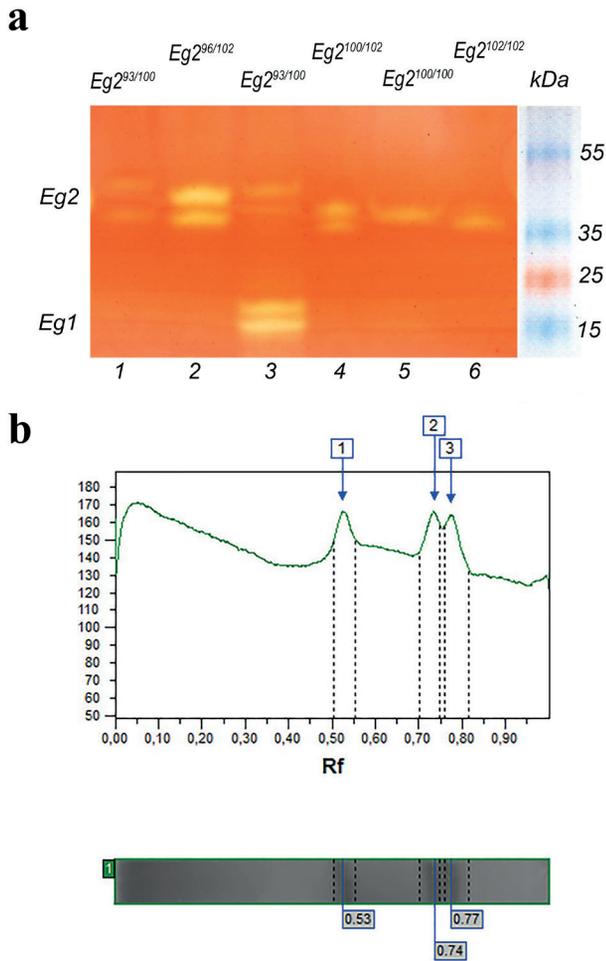
Investigated populations were marked with the name of the closest major settlement.

## Results and Discussion

Data processing revealed that intracellular endoglucanase synthesis in *S. commune* is connected to two genetic loci: *Eg1*, *Eg2* (Fig. 1a). Locus *Eg1* in monokaryotic cultures is visualized in form of two bands with relative mobility (Rf) of 0.74 and 0.77 (Fig. 1b). These may well be two closely linked loci by analogy with the enzyme system of superoxide dismutase in this fungus (Boiko 2015).

Moreover, visualization of the *Eg1* locus is not stable for the most part of dikaryotic cultures (Fig. 1a), it may be illustrative of its dominant type of inheritance and impropriety for population genetic researches. In case of locus *Eg2* we determined four alleles with Rf of 0.53 (93), 0.55 (96), 0.57 (100), and 0.58 (102) (Fig. 1a) and stable visualization regardless of the nuclear status of mycelium.

Studying of genetic diversity of the enzyme system of *S. commune* on the territory of Ukraine with the use of PopGen32 software allowed us to determine that the mean effective number of alleles ( $A_E$ ) is 1.27, Shannon's index of genetic diversity was high (I = 0.43). Considering the populations, the biggest contributions are made by *Pop2* (I = 0.57), *Pop3* (I = 0.39) and *Pop5* (I = 0.39). Contributions in this data are made by dissimilarity of samples, properties of climatic (e.g., humidity, wind streamline) and anthropogenic factor (e.g., density of traffic roads), which affect the speed of propagation of biological objects (Garcia et al. 2014). Migration processes increase the diversity of populations and cause changes in gene frequencies. Frequency of alleles of the locus *Eg2* that are peculiar to the investigated geographic area are show in Figure 2. The dominant role of *Eg2*<sup>100</sup> is noticeable, its frequency is 0.88, the rarest were the alleles *Eg2*<sup>93</sup> and *Eg2*<sup>96</sup>. It should be noted that allele *Eg2*<sup>96</sup> is seen only in population *Pop2*, where allele *Eg2*<sup>93</sup> is completely absent. In case of populations *Pop1*, *Pop4*, *Pop6* and *Pop7* frequency of allele *Eg2*<sup>100</sup> are 1. In case of locus *Eg2* of *S. commune* in separate populations, and in general in the territory of



**Figure 1.** Endoglucanase allozymes of dikaryotic (a) and monokaryotic (b) cultures of *S. commune* (1-6 – patterns of different dikaryotic strains).

Ukraine, we observe deficit in heterozygous genotypes except for population *Pop5* (Table 1). This is also shown by values of Wright's fixation index (Table 2). Such situation may be observable in the absence of random mating in the population which leads to increase in homozygosity in general.

In case of locus *Eg2* of *S. commune*, deviation from the balance of Hardy-Weinberg was found. Such situations are quite frequently observed in populations of fungi

**Table 1.** Observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity of the locus *Eg2* in the investigated *S. commune* populations.

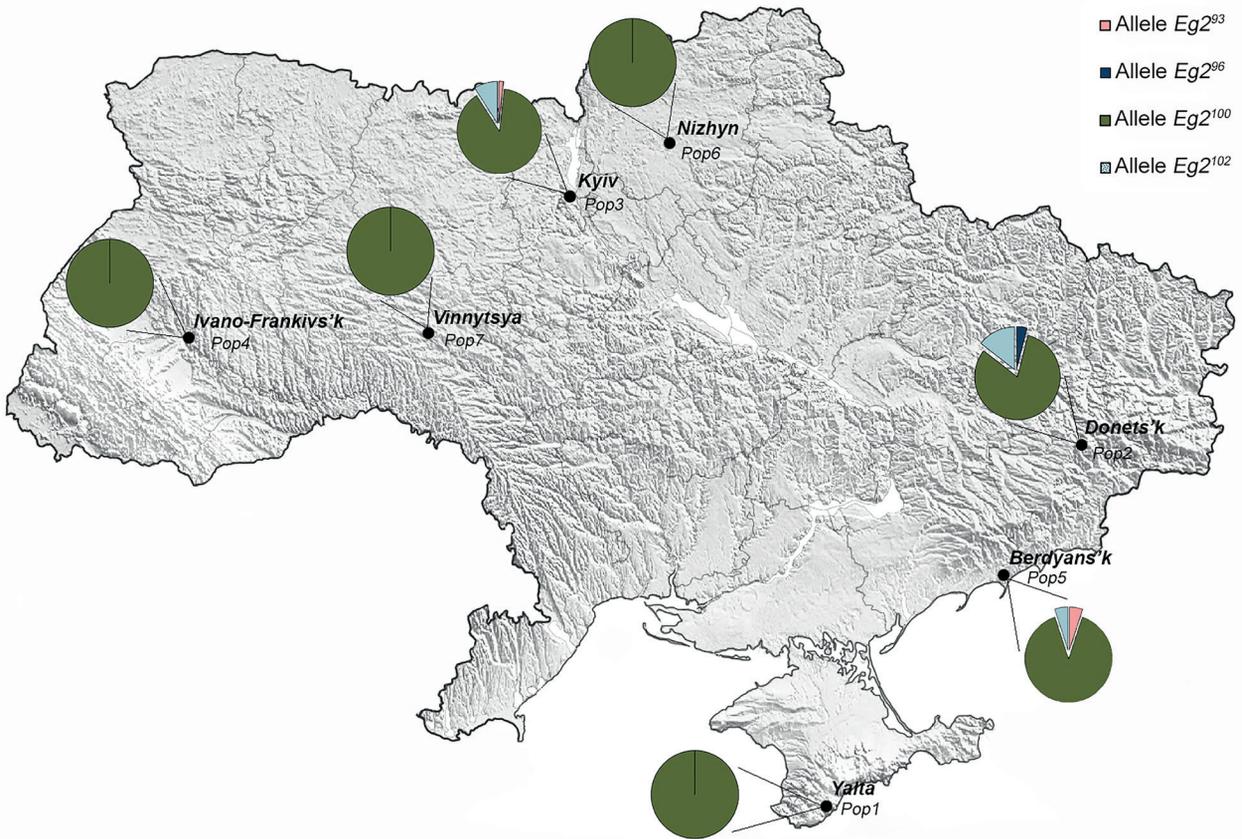
Populations	$H_o$	$H_e$
General	0.0394	0.2139
Pop2	0.0263	0.3161
Pop3	0.0364	0.1977
Pop5	0.2000	0.1947

**Table 2.** Wright's fixation index of the locus *Eg2* in the investigated *S. commune* populations.

Allele	locus <i>Eg2</i>
<i>Eg2</i> <sup>93</sup>	-0.0120
<i>Eg2</i> <sup>96</sup>	0.6627
<i>Eg2</i> <sup>100</sup>	0.8488
<i>Eg2</i> <sup>102</sup>	0.9080

and may have different causes (May and Royle 1982; James et al. 1999; Maurice et al. 2014). Thus, in case of a fungus *Serpula lacrymans* deviations in the balance of Hardy-Weinberg were observed both in European and Japanese populations in form of anomalously high level of heterozygosity (Engh et al. 2010). No clear explanation was found, but there have been suggestions of non-random mating or presence of more than two nuclei in the fungal mycelium. We should also consider the complex tetrapolar system of sexual compatibility that is peculiar to both *S. lacrymans* and *S. commune* and reduces inbreeding. In our case, domination of one allele, *Eg2*<sup>100</sup>, and low level of heterozygosity of the locus in Ukraine in general (Table 1) could indicate inbreeding in the *S. commune* population. However, data obtained previously regarding other *S. commune* loci indicated abundance by the Hardy-Weinberg principle; and this excludes a significant role of inbreeding in population-genetic processes that take place in this fungus. Obviously, results obtained on one locus only cannot be used as a basis for conclusions regarding the state of populations of *S. commune* particularly or in general in Ukraine, but they raise a question on the causes of the irregular distribution of alleles and give a chance to assess the evolution of this enzyme system. It is fair to assume that alleles *Eg2*<sup>93</sup>, *Eg2*<sup>96</sup> and *Eg2*<sup>102</sup> are the "youngest" and, in spite of that, they are concentrated only in some populations in the investigated geographic area. It should be considered that, under stable environmental conditions, selection for decrease in recombination can take place, while under unstable conditions, variation necessarily increases which can lead to appearance of new alleles.

Several authors say that expression of two forms of endoglucanases is peculiar to the species of *S. commune* (Paice et al. 1984; Willick and Seligy 1985). The former determined that molecular weight of extracellular endoglucanases varied within the range of 38-40 kDa. The determined partial amino acid sequence (of 39 amino acids) allowed to define similarity of the two enzymes and assume that *EG2* is a proteolytic product of *EG1*, modified during transport out of the cell. So, most probably, the two are related to the expression of one form of endoglucanase. This data correlates to ours. Taking



**Figure 2.** Alleles frequency of the locus *Eg2* *S. commune* on the investigated geographic area.

into account that all isoforms of endoglucanase *Eg2* of *S. commune* that we described had a molecular weight of 35 to 40 kDa (Fig. 1), we used this amino acid sequence as a basis for identification. In order to search for homotypes of the initial amino acid sequence (AAECSGATKFDYF-GVNESCAEFGNQIPGVKNTDYTWPS) (Paice et al. 1984) the BLAST software was used (Altschul et al. 1997).

glycoside hydrolase family 5 protein [Schizophyllum commune H4-8]  
 Sequence ID: XP\_003031634.1 Length: 333 Number of Matches: 1  
 > See 1 more title(s)

Score	Expect	Method	Identities	Positives	Gaps
85.9 bits(211)	6e-23	Compositional matrix adjust.	39/39(100%)	39/39(100%)	0/39(0%)
Query 1	AAECSGATKFDYFGVNESCAEFGNQIPGVKNTDYTWPS		39		
Sbjct 25	AAECSGATKFDYFGVNESCAEFGNQIPGVKNTDYTWPS		63		

**Figure 3.** Results of the search and alignment of the amino acid sequence of *Schizophyllum commune* in the NCBI database.

As a result, we identified the 333 amino acid sequence of the enzyme of glycosidase (family 5) that is present in the database of the NCBI (XP\_003031634.1) and is peculiar to

*S. commune*. Level of identity of the amino acid sequence was 100% with no gaps (Fig. 3).

According to data from the International Union of Biochemistry and Molecular Biology (<http://www.chem.qmul.ac.uk/iubmb/>) glycosidase have more than 200 groups of enzymes that differ in activity on substrates. Classification by substrate specificity does not consider evolutionary processes, this is why the preferred classification today is CAZY, that was based on the structural similarity of proteins and that forms families with the similar substrate specificity (<http://www.cazy.org>) (Henrissat and Bairoch 1993). Glycoside hydrolase family 5 (GH5) includes enzymes with such activities as endoglucanase,  $\beta$ -mannanase, exo-1,3-glucanase, endo-1,6-glucanase, xylanase, endoglycoceramidase. The main peculiarity of this family is that one of the conserved regions contains a glutamic acid residue which potentially takes part in the catalytic mechanism (Py et al. 1991). It should be noted that, according to data from the database of the Universal Protein Resource (UniProt) (Apweiler et al. 2004), the total number of glycoside hydrolases in *S. commune* is over 230, and the only sequence of XP\_003031634.1

10	20	30	40	50
SNSAPQGQAP	SSTATAPAAT	ASSAAAECSG	ATKFDYFGVN	ESCAEFGNQN
60	70	80	90	100
IPGVKNTDYT	WSPSSIDYF	MQDGLNSFRI	PFLLERLAPP	ASGITGDFDS
110	120	130	140	150
TYLADLQSTV	EYITGKGGYA	IIEPHNFMII	NGSTINSPE	FASFWTNLAT
160	170	180	190	200
LFIDNTNVIF	DLMNEPHDID	ASVVYEHMQA	AVNAIRAAGA	TEQLILVEGT
210	220	230	240	250
SWGAWTWIT	SGNSDAFAAL	TDPNDNIAIE	MHOYLSDSGS	GTNETCVSAT
260	270	280	290	300
IGSERLQAAT	EWLKANNQRG	FLGEMGAGSN	ADCISAVSDA	LCYLQQQEGT
310	320	330		
WIGALWVAAG	PWWGDYFTSI	EPPSGVALSD	IYP	

**Figure 4.** The amino acid sequence of the locus *Eg2* *Schizophyllum commune* (UniProtKB - D8Q439).

(UniProtKB - D8Q439) showed complete homology. We can affirm with a high degree of probability that the amino acid sequence found corresponds with one of the alleles of the locus *Eg2* observed by us (Fig. 4).

The molecular weight of the protein (D8Q 439) is 35.660 kDa, which is in agreement with our data (Fig. 1). Research of the amino acid sequence of all allozymes of *Eg2* *S. commune* will allow us to examine evolutionary changes of the locus.

## Conclusions

In the basidiomycete fungus *S. commune* we found two loci of endoglucanase (*Eg1*, *Eg2*) on the territory of Ukraine. Polymorphous locus *Eg2* controls the expression of four alleles and despite the low level of heterozygosity on the sampled geographic area in general, it showed perspectives of use in population genetic researches. Alleles *Eg2*<sup>93</sup>, *Eg2*<sup>96</sup> and *Eg2*<sup>102</sup> are rare and peculiar to certain populations. The use of a significant amount of *S. commune* samples allowed us to identify the *Eg1* locus, which could not be identified earlier, with molecular weights within the range of 15-18 kDa. A probable amino acid sequence, encoded by the *Eg2* locus, was identified in databases of NCBI (sequence XP\_003031634.1) and UniProt (D8Q439), which is classified among the family 5 (GH5) and consists of 333 amino acid residues.

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