

# Diversity of Ectomycorrhizal Fungi in Young Pedunculate Oak Stand from Morović, Serbia

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## ABSTRACT

Although oaks belong to the economically most important hardwood tree species in Europe, data on the diversity of ectomycorrhizal (ECM) fungi on pedunculate oak in the Republic of Serbia are deficient. The aim of our study was to give the first insight into the diversity of ECM fungi in the young stand of pedunculate oak in Morović, Serbia. The combination of morpho-anatomical characterization and molecular analysis was used for determination of ECM fungi on pedunculate oak. Studied parameters included: (i) counting of vital ECM root tips, (ii) calculation of diversity indices and (iii) classification of morphotypes of ectomycorrhizae into the exploration types. Eighteen ECM fungal taxa were recorded in the studied young pedunculate oak stand. Seven of them were identified to the level of species, eight fungi to the genus, two to the family level, and one ECM fungus remained unidentified. ECM communities consisted of a small number of abundant taxa and a bigger number of rare taxa. The most abundant ECM fungi were *Entoloma* sp., *Thelephoraceae* sp., *Russula* cf. *odorata* and *Russula lilacea*, which made association with the majority of ECM root tips. Short-distance exploration type (ET) dominated, followed by medium-distance smooth ET and contact ET, while long-distance ET and medium-distance fringe ET were rare at the Morović site. Values of diversity indices obtained in the studied pedunculate oak stand were comparable to the ones recorded in different oak stands over Europe. In order to get a deeper insight into the diversity of ECM fungi on pedunculate oak there is a need to continue research on increased number of sites and also to include seasonal dynamics.

**Keywords:** ectomycorrhizae; *Quercus robur* L.; morpho-anatomical characterization; ITS region

## INTRODUCTION

Pedunculate oak (*Quercus robur* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) are among the most economically important hardwood forest tree species in Europe (Ducousso and Bordacs 2004, Eaton et al. 2016, Kesić et al. 2018). They are light-demanding trees that enrich forest biodiversity by affecting the regeneration of many other tree species (Eaton et al. 2016). According to Eaton et al. (2016), fertile and well-watered soils are preferred by pedunculate oak, although adult trees tolerate floodings (Eaton et al. 2016).

In the Republic of Serbia pedunculate oak covers 32400 ha of the land, which makes up to 1.4% of the total forest

area (Banković et al. 2009) with the largest areas in the valleys of the Sava, Danube, and Morava rivers, while the best-quality pedunculate oak forests are situated in the Srem District (Rađević et al. 2020). However, oak trees in lowland forests are under detrimental influence of climate change and pedunculate oak is a species especially sensitive to precipitation and temperature (Kostić et al. 2019).

In most temperate ecosystems, the establishment, growth and survival of forest trees depend on mutualistic association with ectomycorrhizal (ECM) fungi. ECM fungi are successful in the absorption of water and nutrients from the soil and their translocation to plants, while plants provide fungi with carbohydrates (Smith and Read 2008). Common

mycelial networks connect plants of the same and different species with decomposers in the forest soil and can affect the ecophysiology of plants (Selosse et al. 2006). The importance of ECM refers to their supportive role in forest regeneration, succession and resilience to stress factors (Selosse et al. 2006). Particularly, trees that are under environmental stress benefit from the stabilizing effects of mycorrhizas (Milović et al. 2021a, Stojanović et al. 2021).

The functional compatibility of the partners in ectomycorrhiza is species-specific and depends on both partners (Kraigher 1996). Pedunculate oak is the host of Italian white truffle (*Tuber magnatum* Pico) which is the most expensive fungal species in the world, and it cannot produce fruit bodies without its host (Katanić et al. 2017). Not only that knowledge about the structure of the ECM community could give important information about the physiology of forest trees, but it can also reveal a lot about the functioning of forest ecosystems (Kraigher et al. 2011).

Considering the scarcity of the data on the ECM diversity of the oaks in the Republic of Serbia, especially those from underground studies of ECM root tips, this study aimed to give the first insight into the diversity of ECM fungi on pedunculate oak from Serbia.

## MATERIALS AND METHODS

### Sampling Site and Procedures

Sampling was conducted in a 18-year-old pedunculate oak (*Quercus robur* L.) stand (N 44°56'55.5", E 19°10'56.7" and 82 m a.s.l.) situated in forest administration "Morović" under the management of Public Enterprise "Vojvodinašume". Also, *Acer campestre* L., *Cornus sanguinea* L. and *Ulmus effusa* Willd were present with a minor share.

In the meteorological station in Sremska Mitrovica the mean annual temperature for the period 1981-2010 of 11.2°C and the average annual amount of precipitation of 641.64 mm were recorded. Based on the average monthly sum of precipitation in this area, June was the month with the most precipitation, while February was the driest (RHMZ 2021).

Soil sampling was performed at the beginning of July 2018. Six soil samples were taken with a standardized soil corer (Kraigher 1999) with total volume of 274 ml and length of 18 cm. Soil samples were stored in the fridge for up to three months. To loosen the soil structure, each sample was submerged overnight in tap water before analyses. All fine roots were carefully washed from the soil and divided into vital ECM root tips or old, non-turgescence and non-mycorrhizal roots using a dissecting microscope Olympus SZX 10 (Olympus Corp., Tokyo Japan) with magnifications 10-63× (light source: Olympus Highlight 3100, daylight filter). Vital ECM root tips were classified into different morphotypes of ectomycorrhizae based on their morphological and anatomical characteristics, using a dissecting microscope and a microscope (Olympus BX 53®, Olympus Corp., Tokyo Japan) with magnifications 100-1000×. Morphotypes of ectomycorrhizae were described according to the methodology proposed by Agerer (1991) and Kraigher (1996). If it was possible, a fungus from ectomycorrhiza was identified by comparison with descriptions published in Agerer et al. (2006), Agerer (2008), or Agerer and Rambold (2021).

Morphotypes of ectomycorrhizae were categorized into the exploration types as suggested by Agerer (2001). All categories of fine root tips were quantified by counting under the dissecting microscope.

### Molecular Identification of Ectomycorrhizal Fungi

Molecular identification of fungus in ectomycorrhiza was based on PCR amplification of internal transcribed spacer (ITS) region of fungal nuclear rDNA. DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from ECM root tips. DNA extraction of some morphotypes of ectomycorrhiza was unsuccessful and morpho-anatomical identification was not sufficient to determine the ECM fungus. In that case, ECM morphotype was labeled as an "unidentified" type. Amplification reactions were performed using ITS 1F (Gardes and Bruns 1993) and ITS 4 primer pair (White et al. 1990) in Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). The content of PCR mixture and thermal cycling conditions were explained by Milović et al. (2021b). Amplified DNA fragments were separated and purified from the agarose gel using the QIAquick gel extraction kit and QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and then sent for sequencing to Macrogen Europe B.V. The determination of species, genus, or family of ECM fungi was done by comparison of the obtained sequences to those deposited in GenBank (NCBI 2021) and UNITE (Nilsson et al. 2018) databases.

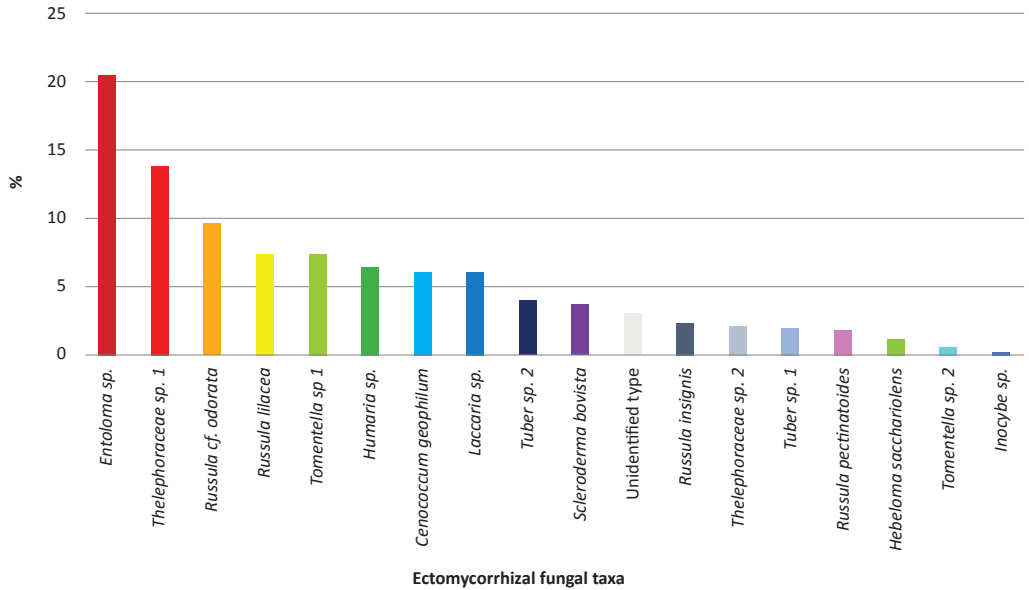
### Data Analysis

Calculation of diversity indexes was performed per sample and per site (i.e., by pooling the data of ECM community) following the formulas given by Atlas and Bartha (1981) and Taylor et al. (2000), which was explained in more detail in the paper by Milović et al. (2021b).

## RESULTS AND DISCUSSION

Eighteen ECM fungal taxa were recorded in the young pedunculate oak stand from Morović, among which seven were determined to the level of species, eight to the genus, two to the level of family and one morphotype remained unidentified (Figure 1).

An analysis of ectomycorrhizas in oak plots across European countries, based on 96 soil cores taken per plot, during one season showed that the average richness was 55, ranging from 24 fungal taxa in the Netherlands to 83 in Romania (Suz et al. 2014). A study of temporal changes in the ECM community of a temperate oak forest in northeast France revealed 75 fungal taxa within 15 months (6 soil cores were taken each month) (Courty et al. 2008). Investigating the diversity of ECM morphotypes and tree decline in two stands of pedunculate and sessile oaks in Austria, Kovacs et al. (2000) recorded 46 and 38 ECM morphotypes within 3 years in a total of 216 soil samples per site. On the other hand, the number of taxa obtained in our research was similar to the ones recorded in two sessile oak stands from Fruška Gora where 17 and 12 ECM taxa were determined in ten soil samples per site (Milović et al. 2021b). Furthermore, in three declining pedunculate oak stands in western Poland the number of ECM fungal taxa per site ranged from 11 to 15 (based on 60 soil samples



**Figure 1.** Relative abundance of ectomycorrhizal fungal taxa (based on the number of ectomycorrhizal root tips belonging to a particular ectomycorrhizal fungal taxon in relation to all analysed ectomycorrhizal root tips) in a young pedunculate oak stand from the Morović site.

taken per site) (Bzdyk et al. 2019). Similarly, investigating the effect of dead wood on ECM colonization in an old growth pedunculate oak forest in north-eastern Poland, Olchowik et al. (2019) recorded fungal species richness in the range from 12 to 14 taxa in 20 soil samples per site. On sessile oak from the Taurus mountains in Central Germany, 18 ECM fungal taxa were recorded (Schirkonyer et al. 2013), while on native oaks (pedunculate and sessile oak) from Ireland 21 ECM taxa were determined in the total of 16 soil samples (O'Hanlon and Harrington 2012). However, the average species richness across three pedunculate oak-dominated woodlands in southern England was 33 and it was in the range from 17 to 45 (Barsoum et al. 2021) (based on 80 soil cores per woodland).

On average, six ECM fungal taxa were recorded in soil samples from the Morović site (Table 1). This is a bit lower in comparison to vital oak trees in Austria (Kovacs et al. 2000) and control stand in *Q. ilex* forest from Spain (de Roman and de Miguel 2005) where on average nine and seven morphotypes were found, respectively. On the other hand, in soil samples from two sessile oak stands from Fruška Gora, only three ECM fungal taxa were found on average (Milović et al. 2021b).

At the Morović site, Shannon-Weaver index with value of 1.4 per soil sample (Table 1) is similar to the one noted in oak stands in Austria where vital trees had this index in the range 1.3-1.5 (Kovacs et al. 2000) and control stand of *Q. ilex* in Spain where it was 1.3 (de Roman and de Miguel 2005). On

**Table 1.** Total and average values of the number of ectomycorrhizal fungal taxa, the number of vital ectomycorrhizal root tips, the number of old, non-turgescient and non-mycorrhizal root tips, % of vital ectomycorrhizal root tips and diversity indices in young pedunculate oak (*Quercus petraea* L.) stand located at the Morović site.

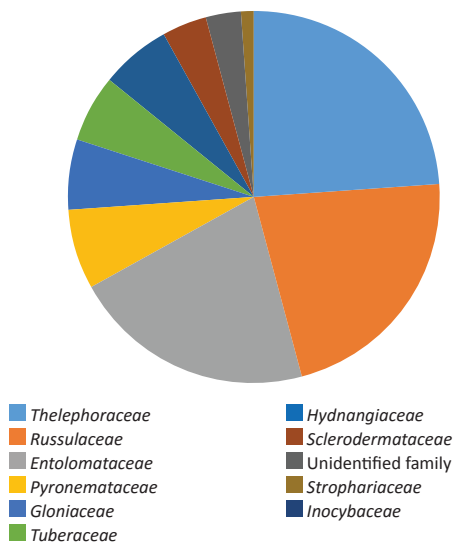
Parameter	Site	Sample
	Total	Average±SE
Number of ectomycorrhizal fungal taxa	18	5.6±0.4
Number of vital ectomycorrhizal root tips	2145	342.4±50.92
Number of old, non-turgescient and non-mycorrhizal root tips	8996	1507.2±406.9
% of vital ectomycorrhizal root tips	19.2	22.5±6
Species richness index	5.10	1.83±0.21
Shannon-Weaver index	2.54	1.44±0.10
Evenness	2.02	1.71±0.08
Equitability	0.88	1.94±0.11
Berger-Parker index	0.79	0.61±0.05

the other hand, the obtained value is higher than in sessile oak stands from Fruška Gora where Shannon-Weaver index was 0.7 and 0.9 (Milović et al. 2021b). Total value of Shannon-Weaver index calculated for site Morović (2.5) is comparable to the one recorded by Barsoum et al. (2021) in pedunculate oak woodlands from southern Britain, which ranged from 1.9 to 2.8. Similar values were noted in Austria for aggregated soil samples taken under vital trees which varied from 2.6 to 2.7 (Kovacs et al. 2000) and in two sessile oak stands from Fruška Gora (2.1 and 2.3) (Milović et al. 2021b).

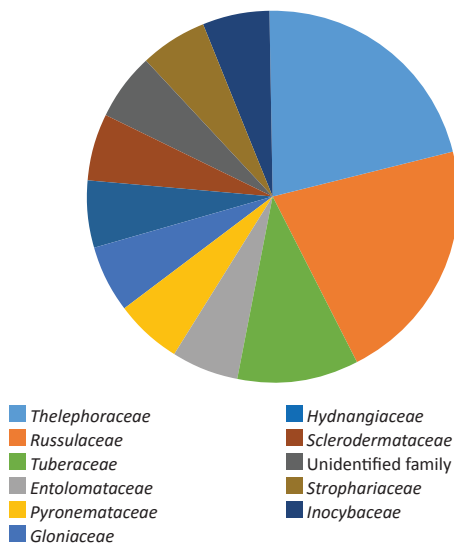
The most abundant ECM fungi at the studied site were *Entoloma* sp., *Thelephoraceae* sp., *Russula* cf. *odorata* and *Russula lilacea*, which together associated with more than a half of all ECM root tips (Figure 1). ECM community was made up of a small number of abundant fungal taxa and a bigger number of rare taxa, which is in accordance with numerous studies (Suz et al. 2014, Rosinger et al. 2018, Bzdyk et al. 2019, Milović et al. 2021b, Barsoum et al. 2021). According to Courty et al. (2008), abundant ECM fungi are usually present all year long, some species occur all year long with fluctuating abundance, while some ECM fungi are characteristic for a particular season or month. Occurrence of a large number of taxa present at low frequencies is important for the stability of the forest stand. If ecological conditions change, some rare ECM species might be adapted to new conditions, while some dominant ECM fungi might not (Pena et al. 2010, O’Hanlon and Harrington 2012).

At the studied site, ECM fungi *Entoloma* sp., *Thelephoraceae* sp., *Russula* cf. *odorata*, *Russula lilacea* and *Tomentella* sp. 1 made associations with the majority of ECM root tips (Figure 1). Their abundances were in the following range: 7-21%. In temperate oak plots across nine European countries, the most abundant ECM fungal species were *Lactarius quietus*, *Cenococcum geophilum*, and *Russula ochroleuca* (Suz et al. 2014). Moreover, *Lactarius quietus*, *Tomentella sublilacina*, *Cenococcum geophilum*, and *Russula* sp. 1 were abundant in a temperate oak forest in northeastern France and present all year long (Courty et al. 2008). The most abundant ECM species in pedunculate oak woodlands in southern England were *Lactarius quietus*, *Cenococcum geophilum*, *Boletus rubellus*, *Tomentella sublilacina* and *Lactarius subdulcis* (Barsoum et al. 2021). *Lactarius quietus*, *Cenococcum geophilum*, and *Tomentella sublilacina* were also found in two sessile oak stands from Fruška Gora (Milović et al. 2021b). *Lactarius quietus* is an oak specialist and one of the most abundant and frequently occurring ECM fungi in temperate oak forest across Europe (Voříšková et al. 2013, Bzdyk et al. 2019). It is interesting to notice that *Lactarius quietus* was not recorded in the studied young pedunculate oak stand at the Morović site as well as any other ECM fungal taxon from genus *Lactarius*. ECM type identified as *Cenococcum geophilum* made association with 6% of all ECM root tips. *Cenococcum geophilum* is regarded as the most widely distributed species complex that makes symbiosis with over 200 tree species (LoBuglio 1999). It has an ability to resist extreme conditions (drought especially), and it is frequently observed on oak trees in Europe (de Roman and Miguel 2005, Schirkyer et al. 2013, Milović et al. 2021b).

*Thelephoraceae*, *Russulaceae*, and *Entolomataceae* were dominant fungal taxonomic families at the Morović site, which colonized more than 65% of all vital ECM root tips (Figure 2). Moreover, families *Thelephoraceae*



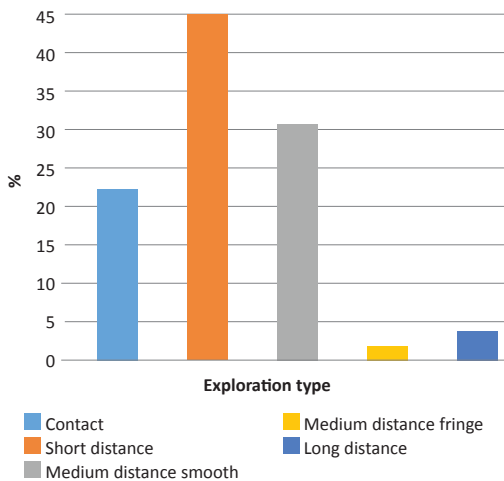
**Figure 2.** Relative abundance of taxonomic families of ectomycorrhizal fungi based on the number of ectomycorrhizal root tips belonging to a particular family in relation to all analyzed ectomycorrhizal root tips in young pedunculate oak stand from the Morović site.



**Figure 3.** Species richness of taxonomic families of ectomycorrhizal fungi based on the number of ectomycorrhizal taxa belonging to a particular family in relation to the number of all determined ectomycorrhizal taxa in young pedunculate oak stand from the Morović site.

and *Russulaceae*, each containing four taxa, were the most species-rich families at this site (Figure 3). Families *Russulaceae*, *Cortinariaceae* and *Thelephoraceae* were noted as dominant in temperate and Mediterranean oak forests (Courty et al. 2008, Richard et al. 2011). In two sessile oak stands from Fruška Gora dominant fungal families were *Thelephoraceae*, *Russulaceae*, and *Inocybaceae* at the Infocenter site and *Entolomataceae*, *Tuberaceae*, *Russulaceae* at the Brankovac site (Milović et al. 2021b). Furthermore, the study of ECM diversity in temperate oak forests throughout Europe reported that the most abundant families were *Russulaceae*, *Gloniaceae*, *Thelephoraceae*, and *Cortinariaceae* (Suz et al. 2014). Similarly, in pedunculate oak woodlands from south England *Russulaceae*, *Boletaceae*, *Gloniaceae* and *Thelephoraceae* were dominant (Barsoum et al. 2021).

Higher number of ECM fungi from the phylum Basidiomycota in comparison to Ascomycota obtained in our study (Table 2) is in accordance with numerous earlier studies (Richard et al. 2011, Suz et al. 2014, Bzdyk et al. 2019, Milović et al. 2021b etc.).



**Figure 4.** Relative abundance (%) of ectomycorrhizal exploration types (based on the number of ectomycorrhizal root tips belonging to a particular exploration type in relation to all analyzed ectomycorrhizal root tips) in young pedunculate oak stand from the Morović site.

**Table 2.** The number of ectomycorrhizal root tips and ectomycorrhizal fungal taxa belonging to Ascomycota and Basidiomycota in young pedunculate oak stand from the Morović site.

	Ascomycota	Basidiomycota
	N	
Ectomycorrhizal root tips	409	1736
Ectomycorrhizal fungal taxa	4	14

According to Agerer (2001), classification of ectomycorrhizae on ETs links fungal morphology (especially the amount and differentiation of extraradical mycelium) with their ecology. Different ETs have different functional characteristics and different capacities for taking up and translocation of nutrients and carbon storage (Hobbie and Agerer 2010). Moreover, ETs can be used for the purpose of site indication, primarily to indicate the composition of soil and the availability of nutrients (Rudawska et al. 2011).

In the studied young pedunculate oak from the Morović site, short-distance ET dominated, followed by medium-distance smooth ET and contact ET, while long-distance ET and medium-distance fringe ET were rare (Figure 4). Mycorrhizae with contact, short- and medium-distance smooth ETs are considered to use labile, mainly inorganic nitrogen (N), while medium distance fringe and mat, and long-distance ETs are assumed to use organic N (Lilleskova et al. 2011, Suz et al. 2014). Furthermore, the long-distance ET, with abundant mycelial biomass was found to be well adapted to nutrient-deficient conditions, being especially deficient in N (Hobbie and Agerer 2010). Investigating ETs of ECM fungi in declining oak stands, Bzdyk et al. (2019) noted that the abundance of contact ET was positively correlated with C:N ratio and organic carbon content, while the abundance of short distance ET was closely related to calcium and phosphorus ( $P_2O_5$ ) content and pH.

Contact or short-distance ETs of ECM fungi are assumed to mostly have wide environmental ranges (Rosinger et al. 2018). Shorter distance ETs are characteristic for colder climates where soils are well provided with total N and plants invest less carbon in mycorrhizal association (Rosinger et al. 2018). On the other hand, in warmer and less fertile environments dominated long-distance ETs of ECM fungi which demand more carbon (Rosinger et al. 2018, Defrenne et al. 2019). A high abundance of shorter distance ETs (contact, short- and medium-distance smooth ETs) recorded in analyzed young pedunculate oak stand from the Morović site suggests that this soil is sufficiently provided with total nitrogen and organic carbon and that therefore nutrients can be reached without bigger carbon investments in extraradical mycelium. Similar results were obtained in sessile oak stands from Fruška Gora (Milović et al. 2021b).

Bzdyk et al. (2019) found the connection between the diversity of ETs and the tree health status, which could indicate potential positive role of ECM functional diversity. Also, a high diversity of ETs could make ECM fungal communities more resilient to environmental change (Suz et al. 2014).

## CONCLUSIONS

This paper presents the first underground insight into the diversity of ECM fungi on pedunculate oak in Serbia. In the studied young pedunculate oak stand from Morović, 18 ECM fungal taxa were recorded, described and identified mostly with molecular methods. The most abundant ECM fungi were *Entoloma* sp., *Thelephoraceae* sp., *Russula* cf. *odorata* and *Russula lilacea* while short-distance exploration type ET dominated, followed by medium-distance smooth ET and contact ET. Values of diversity indices of ECM fungi in the studied pedunculate oak stand were comparable to European research on the ECM diversity in oaks. The obtained results indicate the need to continue research on increased number of sites and to include seasonal dynamics to get a deeper insight into the diversity of ECM fungi on pedunculate oak.

## Author Contributions

MM and SO conceived and designed the research, MM and LK carried out the field measurements, MM performed laboratory analysis, MM and BK processed the data and performed the

statistical analysis, SO secured the research funding and supervised the research, SP, AP and BK helped to draft the manuscript, MM, BK and AGM wrote the manuscript.

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## Conflicts of Interest

The authors declare no conflict of interest.

## Supplementary Materials

**Supplementary File 1** - Ectomycorrhizal fungi recorded in the young pedunculate oak stand from the Morović site identified on the basis of morpho-anatomical characteristics and molecular methods (based on the similarity with the sequences from publicly available nucleotide databases (GenBank and UNITE) and their absolute / relative (%) abundance. Sequences were last compared to nucleotide databases on 15th September 2021.

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