SZENT ISTVÁN UNIVERSITY

DOCTORAL SCHOOL of BIOLOGICAL SCIENCES

PhD THESIS

MYCOLOGICAL INVESTIGATION FROM HUNGARIAN FLOATING ISLANDS

Ágnes Zöld-Balogh

Gödöllő
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Background and objektives

Among the plants- and fungal species of terrestrial and wetland communties tight and multifold networks have been formed in order to maintain life functions. The floating islands swim on the water substrate as the habitats with the thickening peat mats which create transition among terrestrial and wetland habitats. The relationships of plant and fungal communities creating this phenomenon are poorly revealed. In spite of the up-to-date researches their roles have been proved in the biogeogchemical cycles of several elements just like in the avoidance of the fresh water's eutrophication. All of these are strongly connected to anoxic and oxic processes happening in rhizoplane and the storing capacity of peat pointing to the geological future distances. The peat used to cover huge territories of the earth surface which was shrunk into a little region on earth due to the transforming activities of mankind. Artificial floating islands are produced as cleaning 'supplies' for communal and industrial waste water; the role of certain arbuscular mycorrhizal (AM) fungi has been verified in recreation by constructed wetlands following the pollution of grand water.

In order to know fungal communities weaving both sudd plants and peat as a first step the structures of macrofungal associations of certain sudds from the Carpatho-Pannonian Region were analyzed. This was followed by the discovery of the AM fungi relationships in three different types of floating islands related to two Hungarian national parks: floating islands from Lake Velencei, Ráckeve-Soroksári Dunaág's stagnant water in Szigetcsép as well as Lake Fekete in Őrség Region. Disclosing the network systems between plants and fungi of floating islands can be the base of metabolism mapping of this habitat in generally cool and wet microclimate including the decomposition of complex structural polymers.

Our investigations were targeted to:

I. Investigation of macrofungi collected in certain sudds are located in the Carpatho-Pannonian Region

A/ Morphological studies**:** the species-leveled identification was based on morphological examinations.

1. to collect and analyze the floating island Basidiomycetes fungi from two sudd types - sudd covered with *Sphagnum* species and sudd without *Sphagnum.*
2. to compare the distribution of these fungal communities from two sudd types according to habitats and life styles.
3. morphological examinations of Ascomycetes fungi from sudds

B/ Molecular studies:

identify molecularly certain sudd *Hygrocybe* species

II. Investigations of AM fungal symbionts living in sudd's plants from three Hungarian sudds with different trophities.

A/ Morphological studies: analyzes by microscopical and mathematical methods.

Investigation of AM fungi colonization in characteristic sudd plants.

B/ Molecular analyzes to compare the diversity of AM fungal communities from the three habitats studied.

1. phylogenetical analyses of extracted sudd phylotypes
2. ecological analyses of sudd phylotypes
3. analyses of sudd phylotypes were compared with the same phylotypes originated from other habitats.

III. Propose the sudds as unical habitats to protection

Materials and Methods

I./A Morfological investigations of basidiomycetes and ascomycetes communities and their distribution according to life style located in the Carpatho-Pannonian Region/ *Sphagnum* sudds *(Sph)* and sudds without *Sphagnum* (*nSph).*

Basidiomycetes fungi have been collected by the authors - dependent on the circustances of the weather and field conditions - since 1959, while ascomycete since 1994 on the ***Sph*** sudds and n***Sph*** ones. The used technical literature for morphological identification of basidiocarps are: Dennis (1981), Moser (1983), Breitenbach and Kränzlich (1984), Bánhegyi et al, (1985), Brandrud et al, (1989–1998), Schumacher (1990), Lizoň (1992), Courtecuisse et al, (1995), Hawksworth et al, (1995), Hengstmengel (1996), Yao and Spooner (1996), Krieglsteiner (2000–2003), Bratek and Zöld-Balogh (2001), Bellú et al, (2004) and Vesterholt (2005). With the exception of a few species of *Leccinum* genus (Den Bakker and Noordeloos, 2005), as well the *Hebeloma* genus, the taxonomical and nomenclatural categories were applied according to the Index Fungorum (CABI 2008).

I./B Molecular identification of a few uncertain sudd Hygrocybe species

Hygrocybe cantharellus (*H. lepida*), *H. turunda* and *H. coccineocrenata* (the two latter ones proved to be new species in Hungary) their basidiocarps were collected (08.2002; 07.2003) on two *Sph* sudds of the Őrség National Park (Lake Fekete, Lake Sás). The morphological identification (Boertmann, 1995; Candusso, 1997; Krieglsteiner, 2001) was followed by the molecular identification using dried fungi based on ITS sequences.

The extraction of nuclear rDNA and PCR reactions (Gardes et al.1991), the amplifications using ITS1, ITS4 universal (White et al. 1990) and fungi specific ITS1F primers (Gardes and Bruns, 1993) were applied. The cleaning of PCR products was done by Montage-PCRR (Millipore); during the sequence reactions BigDye^{TM} Terminator Cycle 3.1 Sequencing Kit (Applied Biosystems) was used. In the sequencing process of tribes the starting primers were used by ABI PRISM 3100 sequencer. The alignments were made with CUSTALW2 (Larkin et al, 2007) program, while the phylogenetical analyses were with MEGA4 (Tamura et al, 2007).

II./A Uncovering colonization of AM fungi in plants living in floating islands

Root samples were taken from oligothropic Lake Fekete (FEK), mesotrophic stagnant water (RSD) and eutrophic Lake Velence (VEL), respectively from 16, 22 and 26 plant species (3-3 specimen/plant species/3 seasons). The total

roots from herbaceaous plant species and available roots from woody plants were collected from 50x50 m designated areas of habitats mirroring the mosaic patterns of plant associations. On the same areas the pH values from the well water and pressed peat water were measured (10-10 samples from each). Root samples were taken from four rooting levels of sudd plants according to the vertical structure of peat.

The painting method of Krjueger (1968) was modified in order to optimize the visualisation of AM fungal colonizations in sudd plants. The cooking time (in solution of 15% KOH) of roots was adjusted to the plant structure which could last from 1-2 minutes to several hours. Following the roots with high pigment content were shaken for a long time in H_2O_2 solution (30%), occasionally switching solutions several times.

Estimation of colonization parameters according to Trouvelot et al, (1986) using Nikon- Optiphot-2 microscope. The calculated colonization values were stated by Mycocalc program (https://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

Discrimination analysis, Canonical Variates Analysis = CVA method was applied to evaluate the AM fungi estimated colonization parameters on each rooting level among the three habitats and within each sudds respectively (SYN-TAX 2000 program package).

II./B Examination of AM fungal diversity in sudd plants

We extracted DNA with the support of DNeasy Plant Mini Kit from morphologically discovered, colonized roots. The extraction method was slightly modified. This was followed by nested PCR where through sequencing of partial 18S-ITS1-5.8S-ITS2 rDNA region resulted in identification. The first step of PCR was applied universal NS5/ITS4i primers. In the second step seven Glomeromycota specific primers were tested. The cloning was followed by the digestion of the products with restriction enzymes and then RFLP pattern analysis took place. Right after the cleaning of the representative restriction profiles there was a sequencing reaction followed by automated sequencing. The extracted sequences were compared by using BLAST software (http://www.ncbi.nbminh.gov/BLAST/) with the most similar sequences from GenBank database (NCBI) in order to construct phylogenetical trees.

Sequences were aligned using the MAFFT version 7 server (https://mafft.cbrc.jp/alignment/server) and PRANK (Löytynoja A. 2014) with reference sequences of Krüger et al. (2012). Phylogenetic analysis was carried out using the maximum likelihood method by MEGA 7.0.26 with the Tamura-3 parameter model and by RAxML-HPC Blackbox with GTR substitution matrix, final tree was optimized under GAMMA model, available on CIPRES

Science Gateway portal (http://www.phylo.org). Bootstrap analyses were done with 1000 replications. Phylotypes were determined from cladograms as clearly distinct monophyletic taxa. AMF genera or morphospecies associated to each phylotype were assigned on the basis of the position of already known sequences from databases; the nomenclature of AM by Baltruschat et al. (2019) and Corazon-Guivin et al. (2019) was followed.

In order to demonstrate the diversity of phylotypes originated from three floating islands, Shannon diversity index was calculated; PCoA (principal coordinates analysis) disclosed phylotypes distribution among three studied sudds (SYN-TAX 2000 program package).

Results and Evaluations

I./A Basidiomycetes communities from *Sph* and n*Sph* floating islands and their distribution based on their life style

Basidiomycetes

On *Shp* sudd: 76 species from orders of Agaricales, Boletales and Russulales, 15 families and 25 genera; this data originated from 22 habitats and 282 forays.

Highest number of collections: Kis-Mohos (53), Lucs (46), Mohos (44).

Frequency: most frequent: in summer *Galerina paludosa*, *Lactarius helvus* and *Russula emetica;* in autumn: *Leccinum variicolor*; low number of collection number in spring; summer data > autumn data; the basidiocarps were collected during 7 months and 60% of these were found in the 8th and 9th months.

The most frequent mycorrhizal fungi in general: *Lactarius helvus, Leccinum variicolor* and *Russula laccata.*

Most frequent saprobionts: *Hyholoma elogantaum, G. paludosa* and *G. tibiicystis.*

Most frequent substrates: *Sphagnum* species; dominant host plant: *B. pubescens.*

Life style: 66% mycorrhizal, 34% saprobiotic fungi.

On **n*Sph*** sudd: 33 species from the above mentioned three orders + Cantharellales, 18 genera, 17 habitats from 77 forays.

Highest number of collections: Öcs- Lake Nagy (15), VEL (14).

Frequency: low number of species from spring collections but high number of collections;

Autumn data > summer data; half of basidiocarps gathered through 8 months were found during the 9^{th} and 10^{th} months. In general:

most frequent mycorrhizal fungi: *Cortinarius uliginosus, Leccinum scabrum;*

most frequent saprobiont: *Mycena belliae;*

most frequent substrates: *Phragmites*; dominant host plant: *S. cinerea;*

Life style: 55% mycorrhizal, 45% saprobiotic fungi.

Common facts in both types of sudds: mycorrhizal species are dominant; orders: Agaricales, Boletales and Russulales; the most common species are from Agaricales and Russulales orders.

Basidiocarps were not found from 12th until 3rd months.

Common host plants: *Betula* spp., *Salix* spp., *Picea abies* and *Pinus sylvestris*.

Three common species: *Cortinarius uliginosus*, *Psathyrella typhae* and *Russula laccata.*

Mycorrhizal communities according to sudd regions

1. **On the fringe of sudd**: balanced number of mycorrhizal and saprobiotic species; **dominant species:** *Salix* species; **mycorrhizal fungi species**: *C. cinnamomeoluteus*, *C. helobius*, *C. uliginosus*, *Hebeloma pusillum*, *Inocybe salicis* and *R. laccata.*

2. **Central part of sudds**: dominancy of mycorrhizal species; under *Pinus sylvestris* e.g.: *C. huronensis*, *Suillus bovinus* and *S. variegatus.*

3. **Fringe + central part of sudds**: mycorrhizal character species under ***Betula*** species: *C. pholideus*, *C. tubarius*, *L. cyaneobasileucum*, *L. holopus*, *L. variicolor* and *R. betularum*; under ***Picea abies*** the most common species: *C. subtortus* and *R. emetica.*

Ubiquist basidiomycetes: *Laccaria proxima* (under *Betula*, *Salix* and *Pinus* species); *Amanita fulva*, *C. semisanguineus* and *Paxillus involutus.*

The ratio of mycorrhizal and saprobiotic species from Hungarian floating islands is similarly balanced compared to mires data in Finnland (Salo, 1993).

Ascomycetes

On n*Sph* spots from sudds (exception of *Mitrula paludosa*): 27 species from Dothideales, Helotiales, Orbiliales and Pezizales orders, 11 families, 19 genera, 13 habitats from 69 forays.

Frequency: data only from 3rd until 11th months. Most frequent: in 10th and 6th months; the most frequent 1. in autumn: *Mollisia ligni*; 2. in spring: *Scutellinia crinita;* 3. in summer: *Geopora tenuis* and *Lachnum virgineum.*

In general the most frequent ascomycetes (provide half of data): *Geopora tenuis*, *Lachnum virgineum*, *Mollisia ligni*, *Orbilia luteorubella* and *Scutellinia crinita*.

Most frequent mycorrhizal: *Geopora tenuis.* Most frequent saprobe: *Scutellinia crinita.*

Most frequent substrate: 70% rotten wood/mostly *Salix* sp.; 19% decaying stem and leaves of herbaceous plants; 3% soil. Dominant host plant: *S. cinerea.*

Life style: 92% saprobionts; 8% conditional mycorrhizae-producer: *Geopora tenuis* and *Morchella elata*.

On floating islands up until now sudd specialist ascomycetes have not been found.

I./B Molecular identification of uncertain sudd *Hygrocybe* species

Following the molecular analysis (total ITS1 + 5,8S rDNS + ITS2 regions) of three sudd *Hygrocybe* species phylograms were built. Two successfully sequenced species samples proved to be the same taxon of Pseudohygrocybe suborder. This clade includes taxa of which basidiocarps macroscopically can be characterized with vivid yellow, orange and red colors; covering sequences of Lepida aggregate of Squamulosae subsection (Coccineae section) as well. The clade of two samples forms a distinct clade from *H. miniata* aggregate's clade with adnexed lamelles. In between, *H. cantharellus* (*H. lepida*) and *H. turunda* taxa (pileus covered with tiny scamuloses, decurrent lamelles) significant microscopical difference was not verified. In this clearly stand-alone clade, these two taxa seemed to be identical on the phylogenetical tree.

II./A Colonization of AM fungi in sudd plants

Sudd water trophity/pH-values, colonized plant species: there are characteristical differences among pH-values from oligotrophic - acidic FEK, mezotrophic - nearly neutral RSD and eutrophic - more alkaline VEL. Besides, the pH-values measured in surface well waters proved to be always higher than the data originated from peat water pressed in the same floating island.

The number of colonized plant species is in direct ratio to the level of habitat trophity/pH-values on all three floating islands. The plants proved to be colonized in the following ratios: in oligotrophic sudd from 16 plant species only 9, in the mezotrophic one from 22 plant species 17, while from the eutrophic sudd's 25 plant species 24 were colonized.

Plant species building rooting levels: while the woody plants, deeper rootings and rooted on surface species (from the II., III. and IV. rooting levels of sudds) proved to be dicots with the exception of *Eriophorum angustifolium* (Cyperaceae) from FEK and *Calamagrostis canescens* (Poaceae) from VEL, but the sudd constituents (I. rooting level) can be exclusively grouped to the monocots on all three studied sudds.

Visualization of colonization: the clearing in hot KOH solution of roots was adjusted according to the characteristics of sudd plant species avoiding extraction of AM fungi from the roots. Due to the significant quantity of pigments in roots we did very thorough decolorization (H_2O_2) almost in case of all sudd plant specimens. Following the decolorization the dye ensured the exact estimation of AM fungi's structure and colonization.

***Arum* and *Paris* phenotypes of AM fungi colonization:** the *Arum*-type structure of AM fungi was introduced by a photo about root cortex cells of *Frangula alnus.* In this the structure hyphae move through the intercellular space and intrude into the plant cells and create branching arbuscules in a single cell. In the contrary, intracellular hyphae of *Paris*-type of AM fungi colonization, move from one cell to another in peripheral root cortex cells and create a large number of coils and arbuscules. This has been verified by the picture taken of AM fungi colonization in *Lysimachia vulgaris.* While semi-preserved preparations of roots originated from summer samples show diversely branching structures, the semi-thin and ultra-thin preparations indicate degraded structures of AM from autumn vegetation period.

Colonization of habitats/rooting levels: in the peat of mezotrophic and eutrophic sudds the presence of AM fungi was disclosed from all four plant rooting levels. On oligotrophic floating island only the plants of the lower three rooting levels proved to be colonized. On its surface rooting plants AM deficiency showed with higher colonization parameters in its I. rooting levels simultaneously and differing from the two other habitats which are richer in nutrients (confirmed also by CVA). The II. and III. rooting levels of colonization from the three sudds are similar The colonization of oligotrophic sudd's I. rooting level proved to be significantly higher than the colonization levels of the same rooting levels from the two other floating islands.

Colonization of plant species: strongly colonized: the woody *Frangula alnus* (F=72-100%; A=4-77%) of II. rooting levels; the herbal *Lysimachia vulgaris* (F=71-100% A=3-60%) from III. rooting levels. The also common, ectendomycorrhizal *Salix* species (F=10-90%; A=0-10%) showed low but continuous level of colonization independently from the seasons. In the I. rooting level of oligotrophic habitats plants *Molinia arundinacea* stands out with the highest colonization estimates. In the same habitat the *Carex* species examined can be characterized with higher colonization estimates than the *Carex, Typha* and *Phragmites* species from two other sudds.

By applying our own visualization method the presence of AM fungi symbionts were discovered in the representatives of *Cyperaceae*, *Poaceae* and *Typhaceae* living on floating islands in all three seasons.

The specimens of colonized sudd plant species by AM fungi showed continuously a certain level of colonization in the studied, three seasons.

Deficiency of AM fungi colonization: FEK: *Betula pubescens, Drosera rotundifolia, Menyanthes trifoliata, Picea abies, Pinus sylvestris, Polytrichum commune, Populus* sp., *Quercus* sp., *Sphagnum palustre*, *Sph. recurvum* és *Sph. subsecundum*; **RSD**: *Pellia endiviaefolia, Sph. fimbriatum, Sph. squarrosum, Sph. teres* és *Thelypteris palustris*; **VEL:** *Th. palustris* – these species showed total lack of AM fungi colonization.

II./B Diversity of AM fungi in sudd plant species

DNA extraction: according to the original recipe: the DNA extraction from 2, 3 x 1 cm root pieces/samples (randomly chosen) barely provided results in the case of sudd plants. Following our new method the DNA extraction - from 11 x 1 cm root pieces/samples - resulted in successful sequencing in the vast majority of the cases.

PCR, clones: out of the seven Glomeromycota specific primers tested only three (ACAU1661, LETC1677 and GLOM1310) resulted sequences applying together with ITS4i. The summarized success ratio of these primer pairs (148 PCR products) was 64%, out of which 21% (31 PCR products) provided Glomeromycota clones. The distribution of latters: 65 clones from **9** FEK samples, 56 ones from **10** RSD samples, while 98 positive clones originated from **12** VEL samples which were verified by the analysis of RFLP pattern.

Phylotypes: all 77 DNA samples were isolated from 8-8 plant species of each three floating island. **15 sequence phylotypes** were identified, out of which **9** related to **Glomeraceae** (GLO), **4** belong to **Entrophosporaceae** (ENT) and **2** to **Acaulosporaceae** (ACA) families. Phylotypes of the GLO family are present in the highest number in all three sudds however the phylotypes of the other two families are distributed between two habitats. Lack of the ENT representatives is shown only on oligotrophic FEK while ACA phylotypes are missing only from mezotrophic RSD.

The highest level of phylotype diversity showed on VEL by 9 phylotypes compared equally to 4-4 phylotypes from the other two floating islands. Among 15 phylotypes only two proved to be common on the nutrient-richer floating islands. The uniqueness of FEK phylotypes was verified.

FEK phylotypes: A-2, A-4, A-5 and C-1; RSD phylotypes: **A-1**, A-6, **A-8** and B-2;

VEL phylotypes: **A-1**, A-3, A-7, **A-8**, A-9, B-1, B-3, B-4 and C-2 (common phylotypes are indicated with bold fonts).

Calculated Shannon diversity index on three floating islands: 1,19 on FEK, 1,24 on RSD and 1,68 on VEL; while the whole study showed 2,22 diversity index. PCoA presents different AM fungi community on oligtrophic floating island and the overlapping fungi communities between mezotrophic RSD and eutrophic VEL.

Floating island phylotypes homologue to the closest AM fungi species:

A-I. *Rhizoglomus intraradices*/*fasciculatus* group; **A-III.** *Rhizoglomus proliferus*

A-VI. *Dominikia aurea*; **A-VIII**. *Septoglomus constrictum*

B-III. and **B-IV.** *Claroideoglomus claroideum*

C-I. *Acaulospora koskei*; **C-II.** *Acaulospora mellea/delicata*

Oligotrophic FEK differences proved by: 1. Absence of I. phylotype (generalist!) on oligotropic FEK (this A-I. phylotype is the most widely spread on the VEL and FEK as well = 71 clones from the whole 219 clones); **2.** Sequences from sudd constituents (I. rooting level) only from FEK were disclosed **3.** presence of AM fungi was not revealed in plant species of IV. rooting level of FEK **4.** C-1 phylotype related to *Acaulospora koskei* was isolated only from FEK.

Host plant preferences: specimens of *Lysimachia vulgaris* from all three sudds showed high levels of colonization accompanied with the highest value of phylotypes (7) diversity of floating islands. This value can go higher based on absence of molecular results from mezotrophic sudd specimens. Similarly, *Solanum dulcamara* (5 phylotypes) and *Mentha aquatica* (5 phylotypes) which show as hosts of high-numbered phylotypes in spite of their specimens missing from oligotrophic habitat. Only the representatives of ectendomycorrhizal *Salix* genus common in all three habitats provide phylogenetical information about the three sudds. *Salix* specimens by four phylotypes also proved to be preferred host plants equally on the three habitats with distinct trophity. The detected phylotypes number from the other sudd plant species is between 1 and 3.

New scientific results

- I revealed the arbuscular mycorrhizal (AM) colonization of 25 characteristic plant species from some rooting layers in three floating islands with distinct trophity in Hungary
- I isolated 77 DNS samples from the roots of 14 sudd plant species; out of this I identified 15 sudd sequence phylotypes.
- I verified the diversities of the AM fungi assotiation's in three main sudd types of Hungary.
- I certified, that our 15 sudd phylotypes is closely similar to phylotypes from other extreme habitats.
- I demonstrated a permanent presence of AM fungi colonization in individuals of certain Carex-species and Eriophorum angustifolium in sudds.
- I documented the separation of the habitats and rooting levels on the basis of AM fungi colonization in plants individuals.
- I identified by molecular method two Hygrocybe taxa which were detected exclusively on sudds in Hungary

Consequences and suggestions

Our results have established further discovery of ascomycetes, basidiomycetes/ectomycorrhizae and AM fungi communities living in Hungarian floating islands. Firstly, the reveal of species spectra by molecular methods could be important in the process of understanding the biological significance of floating islands. This is strengthened by almost 100 sudd basidiomycetes samples in the author's fungarium which need molecular taxonomical analysis (on morphological basis they cannot be identified).

The taxonomical re-evaluation of *Hygrocybe* genus is ongoing by the identification of floating island's taxa applying molecular methods. Based on the above written and presumed identification of *H. coccineocrenata*, molecular and phylogenetical analysis needs to be redone once it is fruiting again.

The sudd plants proved to be colonized by AM fungi, simultaneously as well as in several rooting levels following the layered structure of peat. Further and widespread analyses are needed by the following phenomena: the total absence of AM fungi in surface-rooting plants of oligotrophic sudd; in sudd constituents AM fungi vaguely appearing dynamics as an invert ratio between peat water pH-values and number of arbuscules. Different phylotypes detected from three distinct floating islands could provide a solution to avoid certain environmental damages.

The floating islands represent a transition between terrestrial and wetland habitats, meanwhile their numbers are drastically dropping. The outstanding conservational significance of sudds is strengthened by own research results as well. According to these results in Hungary, the following species were detected exclusively in sudds, up to date: *C. tubarius*, *C. uliginosus*, *H. coccineocrenata*, *I. lacera* var. *helobia*, *Omphalina gerardiana*, *R. betularum* and *R. laccata*. Verified by the above, floating islands provide last asylum for several rare and unique species.

List of publications providing basis of thesis

Published papers in referred journals

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