A new research group of lichen symbiosis

Project proposal

1. State-of-the-art and objectives

Symbiotic relationships are all around us. They increase biodiversity by facilitating diversification within ecological niches that would otherwise be inadequate (1). It has been hypothesized that symbioses played a crucial role in the diversification of early eukaryotic life, and were responsible for the colonization of land by plants ca. 450 million years ago (2). Of all symbiotic associations, trophic mutualistic symbioses are among the most important. In these interactions, both partners receive a benefit of resources, i.e. nutrients and energy to survive (3). The energy is usually provided by a plant or a photoautotrophic microorganism. Examples of obligate trophic symbioses, in which both partners are completely dependent upon each other, include associations between mycorrhizal fungi and orchids, between corals and zooxanthellae, and between fungi and algae in lichen associations (4). The latter two examples represent stable, ecologically dominant mutualistic associations in marine tropical and terrestrial arctic environments, respectively (5).

However, very little is known about the mechanisms underlying the selection of partners in newly developed symbiotic associations. In lichens, photobiont switching (algal transfer among fungal partners) by ascospore dispersal and thallus re-establishment has been reported for a number of species (6). Both photobiont specialists and generalists have been distinguished in many lichen genera (7). In addition, specific PCR techniques demonstrated the coexistence of different algal species in a single lichen thallus (8), thus facilitating the proliferation of this lichen across a wide range of habitats. Recently, algal switching has been reported in coral symbioses, as well (9). A symbiotic host may respond to changing environmental conditions by habitat-adapted symbiont association, a process known as a coral probiotic hypothesis (10; 11). However, the role of algal switching in determining the symbiotic association distributions is entirely unknown, as is the commonness of several symbiotic partners coexisting in a single host tissue.

Just a few weeks ago, a new discovery drastically changed our understanding of the most common terrestrial symbiotic association, the lichens. For nearly 150 years, this model organism of symbiosis was thought to be composed by a fundamental partnership of ascomycete or basidiomycete fungi and photosynthetic phycobionts. Now, researchers have uncovered an unexpected obligate third partner embedded in the cortex of ascomycete macrolichens, basidiomycete yeast (12). This finding will considerably alter our understanding of lichen symbiotic associations, and could explain several yet unaccountable issues, e.g., the genetic uniformity of morphologically different lichen species or the impossibility of artificial lichen synthesis in the laboratory. Now, we have an exciting possibility to study, for the first time, the lichen symbiotic association in its complex nature, considering all three symbiotic units.

The proposed project aims to understand the very nature of mutualistic symbioses by examining equally the distribution, ecology, selectivity and compatibility of the three lichen symbiotic partners, an ascomycete fungus, photosynthetic alga, and a yeast. We will use the lichen genus *Cladonia* as a model organism, applying the comparative, multi-tools, and multidisciplinary approach. To obtain a complex insight into the nature of lichen symbiosis, we will in particular focus on three specific objectives: 1) examining the distribution and ecological preferences of symbiotic partners; 2) revealing the selectivity potential of symbionts; and 3) investigating the compatibility and viability of partners.

Objective 1: Distribution and ecological differentiation of symbiotic partners

The first objective seeks to determine the abiotic factors that drive the distribution of lichen symbionts. This will represent a fundamental and essential step towards unveiling the nature of the lichen symbiosis. We will evaluate the distribution of selected genotypes in the ecologically and climatically divergent European regions, by employing Sanger sequencing with universal fungal, algal, and yeast primers. This approach will filter out any secondary co-existing minor partners. We will ask the following questions:

Q_1. Which abiotic and ecological factors significantly influence the distribution of symbiotic partners?

Q_2. Do the coexisting partners exhibit comparable distribution patterns?

Objective 2: Selection towards the pool of available partners

One of the most intriguing challenges in the biology of symbiotic organisms is to determine the rules of partnership. Very little is known about the mechanisms shaping the associations of lichen symbioses (7; 13). To uncover these selection mechanisms, we will characterize the total diversity of symbiotic partners inside and outside lichen thalli at given localities. We will apply the state-of-the-art next generation sequencing (NGS) to inventory the real diversity of symbiotic partners. We will ask the following questions:

Q_3. How is the available pool of symbionts mirrored in the realized diversity of symbiotic partners?

Q_4. How do the distribution ranges of lichen species correspond to the available pools of symbiotic partners?

Q_5. What is the portion of ecologically redundant partners in lichen communities?

Objective 3: Compatibility and viability of symbiotic partners

Finally, we will assess the compatibility potential of symbiotic partners, and test whether particular partner combinations could broaden the geographic distribution of lichen associations. First, we will assess the partner compatibility by resynthesizing a broad range of associations. Then, we will, for the first time, aim to resynthesize the lichens using all three symbiotic partners, allowing us to overcome the problems related to the unsuccessful reconstructions of lichens *in vitro* (14; 15). Finally, we will transfer artificial association outside their geographic range, to investigate whether the biotic factors play a significant role in shaping the lichen distribution. We will ask the following questions:

Q_6. Is it possible to artificially join the ecologically distinct partners into viable lichen organisms?

Q_7. Are the compatible but atypical partners able to live in conditions known to be suitable for only one of the partners when not in symbiosis?

Q_8. Could we broaden the geographic range of endemic host species by producing artificial lichen associations with ecologically suitable symbiotic partners?

2. Expected scientific output of the project

We propose a research program that has a very high probability of substantially advancing our understanding of the real nature of trophic mutualistic symbioses, and the roles of particular partners in shaping the distribution of symbiotic associations. It seeks to address fundamental challenges in the general understanding of these organisms. The results of the project have the potential to focus and direct conservation efforts. We hope that our findings will open entirely new ways for investigation, conservation, and development programs of these unique, valuable, and generally threatened ecosystems.

3. Methodology

Our methodology includes the *comparative* (7 climatic regions; 56 communities; ca. 1,120 specimens) and *multi-tools* (broad field sampling; niche modelling; Sanger sequencing; amplicon NGS sequencing; translocation experiments; cultivation; partner synthesis) approach, representing a newly developed and innovative pipeline developed to understand the complex mechanisms shaping the distribution and ecological stability of lichen symbioses.

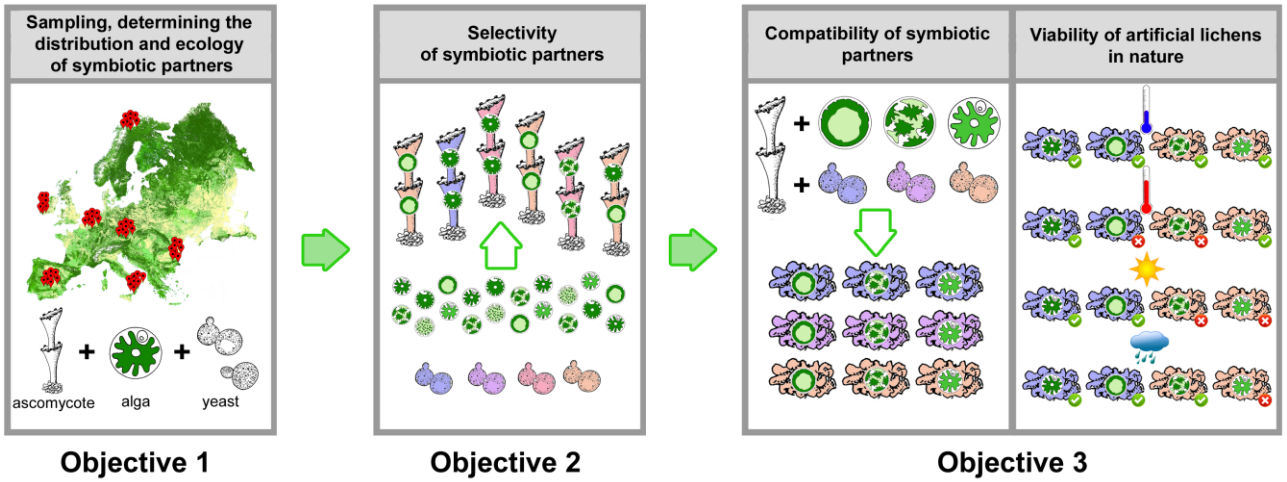

Figure 1. A proposed integrated approach to uncover the role of symbiotic partners in shaping the distribution and ecology of the lichen association (see text below for explanation). Different algal genotypes are illustrated by green cells of various morphologies; yeast genotypes are differentiated by colours.

Objective 1:

We will focus on lichen *Cladonia*, representing a widely distributed European genus containing both algal and yeast partners as symbionts (12). A total of ca. 1,120 lichen specimens will be sampled. The sampling will be conducted at seven climatically distinct European regions (Fig. 1). At each region, 8 sampling sites will be selected to cover the wide range of soil substrates. At each sampling site, ca 20 lichen thalli will be sampled to cover all occurring species. At each sampling site, we will collect ca. 50 g of a soil sample for subsequent soil analyses (16) and NGS investigations (see below).

For Sanger sequencing, genomic DNA will be extracted following the standard CTAB protocol (17). PCR reactions will be performed using specific primers designed to amplify ITS rDNA sequences of each symbiotic partner (18; 19; 20; 12). The phylogenetic trees will be inferred as described previously (18; 19).

Based on occurrence data, chemical analyses of soil samples, and WorldClim bioclimatic variables (21), we will predict the ecological requirements and distribution of particular symbiotic partners by niche modelling. We will apply progressive Bayesian site occupancy models with imperfect detection (22), and several other modelling techniques (23).

Objective 2:

To uncover the overall diversity of symbiotic partners, biodiversity assessment using NGS will be performed on total genomic DNA extracted from both composite lichen samples and soil samples taken at each of the 56 communities. We will amplify the variable V9 region of the SSU rRNA gene and ITS1 spacer. DNA will be amplified with three primer combinations designed to target every partner (24; 12). The PCR products will be purified and quantified using a Qubit 2.0 fluorimeter. The libraries will be produced using the NEXTflex™ 18S ITS Amplicon-Seq Kit for Illumina Library Prep, and multiplexed up to 24 by attaching unique barcodes. The libraries will be sequenced on a Illumina MiSeq sequencing system.

Quality filtering, taxonomic classification and bioinformatic analyses will be done as described in (25). We will employ phylogenetic approaches to cluster amplicon reads (26). Relevant reads will be extracted and further processed by phylogenetic analyses as described above. Finally, we will compare the available (soil samples) and realized (lichen communities) pools of symbionts

at each site, among the sites, and among the regions; as well as the diversity of symbiotic partners within lichen communities as determined by Sanger and NGS.

Objective 3:

For the lichen resynthesis, we will employ the set of fungal, algal and yeast cultures isolated from lichen thalli. The symbionts will be isolated from the thallus fragments and subsequently cultivated as described previously (27; 28). Their identity will be verified by DNA sequencing. We will combine a broad range of fungal, algal, and yeast genotypes, using the methodology described in (29; 30). We will select ca. 4-6 fungal genotypes exhibiting the best potential to form artificial lichens with a broad range of algal and yeast species. Artificial lichens will be transferred to small amounts of sterilised natural substrates, and their growth will be regularly observed.

A total of six localities will be selected to test the viability of artificial lichens *in situ*. At each locality, 24-36 experimental plots will be established. The soil surface will be removed and kept to the laboratory for sterilization. The sterilized substrate will be returned to the original place, together with a triplet of artificial lichens grown *in vitro* on the sterilized substrate. We will prevent lichen grazing by molluscs following (31). The growth will be regularly monitored. After the experiment, identity of symbiotic partners will be verified by Sanger sequencing.

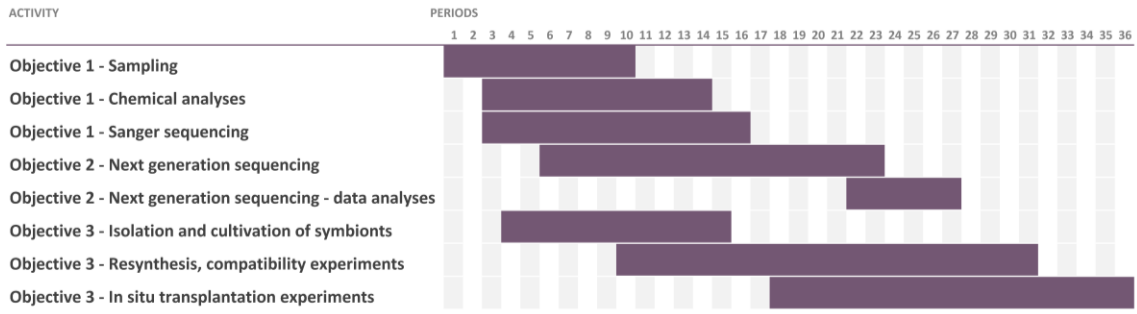

Figure 2. A Gantt chart showing the sequence and timing of the project tasks.

4. Specification of the new group and workplace

The project will be carried out at the Department of Botany, Faculty of Science. At present, the lichenology research topics mostly focus on the traditional disciplines, such as diversity estimations, ecology, and taxonomy of host fungi. However, new discoveries and methodological inventions have opened up new possibilities in lichen research, with a great chance to obtain stimulating novel findings. The workplace is fully equipped to handle the full range of methods proposed. Amplicon NGS will be performed in BIOCEV, Faculty of Science.

This project develops a coherent, innovative and long term research program around questions that are new to the both Department and Faculty. This project brings together experts from different disciplines (phycology, lichenology, genetics, and ecology) to establish a new, interdisciplinary research group on lichen symbiosis. International post-doc, Patricia Moya, represents a key person who developed recently a new NGS pipeline to characterize symbiotic communities in lichens (32).

I am convinced that this new group will significantly boost up the research activity in the field of lichenology, and will have a positive effect on the research skills of students.

5. International co-operation

The project will be carried out in a close cooperation with a recently established international consortium of several lichenology and phycology research teams (incl. Italy, Spain, USA, Germany), focused to intensify joint biodiversity research on lichen symbiosis. In addition, we will continue to collaborate with University of Graz and RBGE Edinburgh in terms of joint publication effort and exchange of MSc/PhD students.

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