

The ecology and distribution of European orchid mycorrhizal fungi

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Part I.

Introduction

The kingdom Fungi is one of the most diverse groups of organisms on Earth. Fungi play an important role in the ecosystem and have a huge impact on biogeochemical cycles, plant and animal pathology, plant nutrition and soil properties. While historically fungi were taxonomically clustered with plants (Copeland, 1938, 1956), towards the middle of the twentieth century it became clear that it failed to properly deal with the differences between the two groups. In 1969 R. H. Whittaker divided the organisms into five kingdoms: Animalia, Plantae, Fungi, Protista and Monera (Whittaker, 1969). By the 1970s this division became widely accepted, and the Kingdom Fungi was recognized. However, due to limitations understanding of the taxonomy, evolution and phylogenesis of fungi was still a matter of ample debate. All the analysis were based on morphological differences, with all of its downsides.

The fossil record for fungi is meager and even though the known fossils cover almost all major fungal lineages (Lücking et al., 2009), it is rather incomplete relative to the evolutionary history of the various fungal lineages. The earliest compendium of fossil fungi is from the late 19th century (Meschinelli, 1898), and their symbiotic relationship with plants in fossils was suggested around that period (Renault, 1896), but the difficulty in the interpretation of morphological data made it impossible to actually understand what happened.

Earliest fossil with the morphological features of a fungus is dated to around 1 billion years ago, and was found in the Arctic Canada (Loron et al., 2019), and there is evidence of fungus-like organisms in fossils of around 800 Mya (Bonneville et al., 2020). Starting from the lower Devonian (around 400 Mya), fossil record is more abundant (Lücking et al., 2009).

It was not until the development of molecular phylogenetics techniques that some light could be properly shed on the evolutionary history of fungi (James et al., 2006a). The molecular clock is one of the most widely used tools to investigate the timing of phylogenetic events. It is based on the hypothesis of the constancy of the rate of evolution with time and, when combined with the use of fossil records, allows the dating

of branching events on phylogenetic trees (Lepage et al., 2007; Weir and Schluter, 2008). From molecular clock analysis seems like fungi are sister group to animals, that is, the two lineages are close, diverging around 1.5 billion years ago (Wang et al., 1999). The two groups form one supergroup called Opisthokonta (Cavalier-Smith, 1987), from the Greek opísthios (rear, posterior) and kontós (“pole” i.e. “flagellum”), since the group is characterized by flagellate cells that propel themselves with a single, posterior flagellum, now lost in most fungi (Steenkamp et al., 2006) with the notable exception of the Chytridiomycota division (James et al., 2006b).

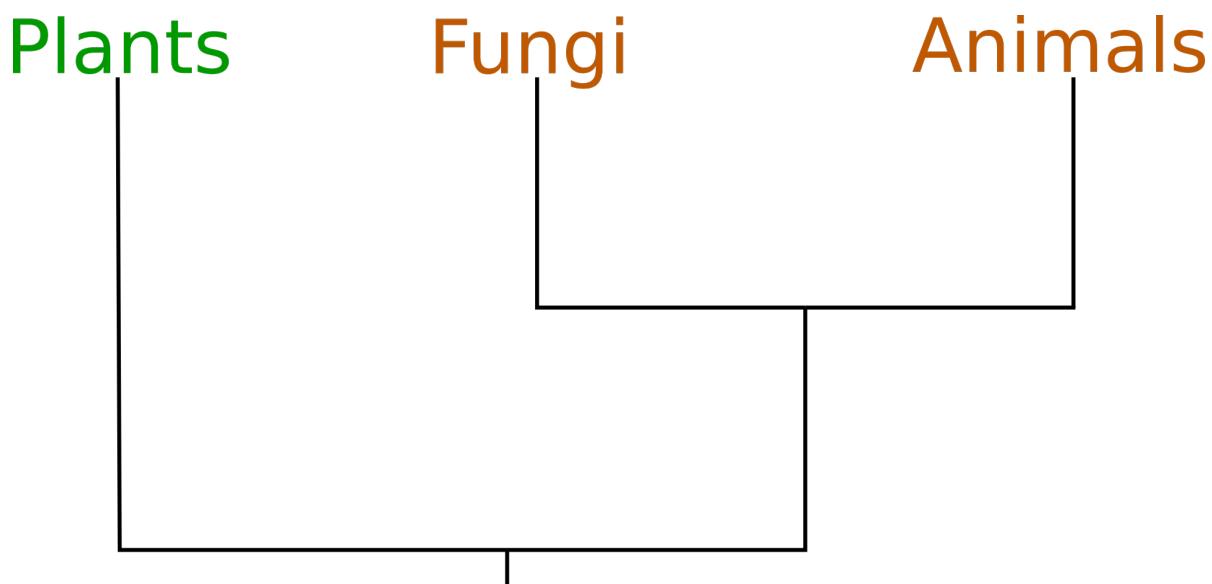


Figure 0.1.: Simplified phylogenetic tree showing the relationship between the three clades

The ancestors of fungi are believed to be simple aquatic forms with flagellated spores, similar to members of the extant phylum Chytridiomycota (chytrids), which are now considered one of the early-diverging clade in the kingdom (James et al., 2006a). The first terrestrial fungi colonized land probably before plants (Heckman et al., 2001), as saprobe (taking nutrition out of dead matter) and/or in symbiosis with organisms capable of photosynthesis. It is commonly accepted that in order to colonize the land, plants had to develop a symbiotic relationship with fungi (Selosse and Le Tacon, 1998; Heckman et al., 2001; Bonneville et al., 2020), but it is not entirely clear whether this

relationship was lichen-like (an algae or cyanobacteria living among filaments of a single or more fungi) (Spribille et al., 2016) or mycorrhizal-like, where the fungus colonizes the host plant's root tissues.

Lichens are the symbiotic relationship between a single or more fungi (*mycobiont*) and a cyanobacteria or algae (*photobiont*). Early plants that transitioned from aqueous environments were poorly equipped to deal with the challenges of a terrestrial life style: mainly, to decompose the mineral substrate to absorb nutrients, to protect itself against dehydration, UV radiations and temperature fluctuations (Selosse and Le Tacon, 1998; Blackwell, 2000). By forming relationships with fungi (i.e. a lichen), the photobiont is protected by the fungal stroma, and it can tolerate drought, cold, heat, intense light and barren rocky substrates. Indeed they also seem to be pioneers of harsh environments today.

Yet, while the relationship between plants and fungi evolved several times (Gargas et al., 1995), the only fungal group we know that are capable of forming such association (called *lichenization*) are Ascomycota and Basidiomycota. The origin of those clades can be dated to about 400 Mya in the Devonian period (Berbee and Taylor, 1993). Similarly there are fossils for lichens dating at the oldest in the Early Devonian (400 Mya) (Taylor et al., 1997; Honegger et al., 2013), while the first fossil land plants and fungi appeared 480 to 460 Mya, and molecular clock estimates suggests about 600–700 Mya (Berbee and Taylor, 1993; Heckman et al., 2001).

Therefore, lichens were likely not what opened the way to plants for land colonization.

The mycorrhizal association is a symbiotic association between plants and fungi happening in the rhizosphere, the plant's root system (Barman et al., 2016). In this interaction there is an exchange of resources between the mycorrhizal fungus and the plant, ideally the plant providing sugar to the fungus and the fungus providing minerals and nutrients to the plant. However, this is not always the case and upon closer analysis, there appears to be a continuum of plant responses to mycorrhizal colonization ranging from positive

to neutral to negative, and the same goes for the fungi (Johnson et al., 1997).

Fossils resembling mycorrhizal relationships date to the Ordovician (with an age of about 460 million years), and are Glomales-like Arbuscular Mycorrhizal Fungi (AMF hereafter), in a moment where the land flora was comprised mainly of bryophytes, pteridophytes and algae (Redecker et al., 2000). Plants can photosynthesize; these fungi can extract minerals from the substrate with great efficiency, protect the root system, extend the range from which water can be taken and protect the plants from pathogens.

Fossil records provides evidence that fungal organisms entered in such symbiosis before the appearance of true roots, and as long as there is a multicellular host AM fungi are fine (Wang and Qiu, 2006; Bonfante and Genre, 2008).

Whether as lichen or as mycorrhiza, the symbiosis between plants and fungi is one of the most important, most ancient relationship in the history of living beings and it surely played a crucial role in the successful colonization of the land by plants (Pirozynski and Malloch, 1975; Malloch et al., 1980; Harley and Harley, 1987; Trappe and Safir, 1987; Selosse and Le Tacon, 1998; Brundrett, 2002). The relationship is so beneficial (for one or both parts) that today is the norm, and is well established in c. 85% of extant plants (Cairney, 2000; Strullu-Derrien et al., 2018), with a high degree of complexity (van der Heijden et al., 2015) and mycorrhizal networks often constitute 20%–30% of total soil microbial biomass (Leake et al., 2011)

1. Orchid-mycorrhizal fungi relationship

Orchidaceae is a diverse and widespread family of flowering plants, containing over 28,000 species in about 736 genera (Christenhusz and Byng, 2016), second only to Asteraceae in terms of species numbers (Ramírez et al., 2007). They are cosmopolitan, with a distribution spanning all continents except Antarctica and including most major island groups (Givnish et al., 2016). By the end of 2017 the IUCN Global Red List included assessments for 948 orchid species, of which 56.5% are threatened (Fay, 2018). In Europe all wild orchids are protected, being included in their entirety on Appendices I and II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CIT) as are many of the habitats they live in, and are listed on the Red List of many countries. Nonetheless, this protection has not staved off a general decline in the orchid flora of Europe (Jacquemyn et al., 2005; Kull and Hutchings, 2006). Major threats include habitat destruction and unsustainable (often illegal) harvesting, and because of their complex life histories orchids are thought to be particularly vulnerable to the effects of global environmental change (Kull et al., 2016; Gale et al., 2018).

Orchids exhibit a high diversity of habitat adaptations, morphologies and pollination strategies, but some characteristics are common to the whole family. One of the most important is the reliance on Orchid Mycorrhizal Fungi (OrM here after) for reproduction and survival. This is because orchids seeds are devoid of nutritional resources, and they completely rely on fungi for nutrition including water, minerals and carbon supply (Leake, 1994; RASMUSSEN and WHIGHAM, 1998; Merckx, 2013) in a nutritional strategy called “mycoheterotrophy”. After germination, seedlings often become autotrophic and subsequently revert to usual mycorrhizal functioning (Rasmussen, 1995; Cameron et al., 2008). Some species, especially from forest environments, remain mycoheterotrophic at adulthood though, developing partial photosynthetic capacity but

1. Orchid-mycorrhizal fungi relationship

still relying on fungi for carbon resources, a nutritional strategy called “mixotrophy” or “partial mycoheterotrophy” (Gebauer and Meyer, 2003; Julou et al., 2005; Selosse and Roy, 2009). Others never develop photosynthetic capacity and therefore rely completely on fungi for nutrition. This nutritional mode, which has evolved over 30 times independently in orchids, is called “obligate mycoheterotrophy” (Merckx, 2013).

While the relationship between orchids and OrM is known for over a century (Bernard, 1899; Rayner, 1927; Rasmussen, 2002; Selosse et al., 2011) and the mechanisms of this symbiosis are beginning to be properly understood, the knowledge from a taxonomical standpoint is still unclear. For many years orchids were thought to interact almost entirely with a specific clade of fungi, members of the Rhizoctonia complex. It was later discovered not only that orchids have way more interactions with different fungi also from the Ascomycetes phylum, but also that Rhizoctonia is a polyphyletic group, and was disassembled in different taxa, all members of the Agaricomycetes, most notably Sebacinales, Ceratobasidiaceae and Tulasnellaceae (Dearnaley et al., 2012). There is also evidence of fungi from the Ascomycota phylum, especially in the order Pezizales (Selosse et al., 2004; Ouanphanivanh, 2008; Waterman et al., 2011), but they are the exception: the most common and known families of OrM are in the Basidiomycota phylum, particularly Inocybaceae, Tulasnellaceae, Ceratobasidiaceae, Russulaceae, Sebacinaceae, Serendipitaceae and Thelephoraceae (Taylor et al., 2004; Roy et al., 2009; Duffy et al., 2019)

1.1. Distribution and ecology of orchid mycorrhizal fungi

Orchids depend on OrM for the germination of their seeds and in many cases for nutrient provision also in adulthood, we assume that OrM must co-occur with the orchid population. However, many OrM can also turn to a soil free-living saprotrophic ecological niche (Oberwinkler et al., 2017) and form mycorrhizal relationships with plants other

1. Orchid-mycorrhizal fungi relationship

than orchids (Selosse and Martos, 2014). Members of the Tulasnellaceae, Ceratobasidiaceae, and Sebacinales (Serendipitaceae and Sebacinaceae) are ubiquitous, with varying relative abundances (Jacquemyn et al., 2017).

Environmental factors that underlie the distribution of OrM taxa are poorly understood. Abiotic variables such as annual rainfall, temperature regimes and soil chemical properties should explain the distribution of many taxa. However, local biotic interaction, community composition and interactions may influence the OrM distribution, especially considering they are symbiotic organisms (Jacquemyn et al., 2017). Overall, there is a lack of evidence, and many parts of the world are undersampled (e.g. all the African continent and most of the tropical areas of the planet). Therefore, it is difficult to conclude that the occurrence of OrM taxa are structured by biotic variables based on a limited amount of data. A part of the problem is that the relationships between orchids and OrM are complex. They both vary in their degree of specialization, from a highly specialized to a more generalist (McCormick et al., 2004; Girlanda et al., 2011; van der Heijden et al., 2015), and OrM may be able to revert to saprophytic free-living lifestyles (Veldre et al., 2013).

In this thesis, I investigate the distribution and ecological factors underlying the occurrence OrM throughout Europe. I analyzed the OrM taxa associated with 16 different orchid species that occur in different regions throughout Europe. Specifically, I focus on two over-arching questions:

1. do similar OrM taxa occur in similar habitats and have similar environmental preferences?
2. Are similarities in OrM co-occurrence explained better by positive environmental correlations or are there residual taxa correlations that may suggest ecological processes beyond environmental filtering (e.g. competition and facilitation)?

Part II.

Materials and methods

Data from bibliography regarding the distribution of OrM in Europe was collected into a starting database. The data had to be of fungi isolated from known orchid roots, and had to be georeferenced at the very least with the name of a close enough place; also, each sample had to have a genbank accession code in order to get the sequences and do the analysis. Only sequences from well-known OrM were considered, that is: Catabasidiaceae, Tulasnellaceae, Inocybaceae, Serendipitaceae, Sebacinaceae, Russulaceae and Thelephoraceae (Dearnaley et al., 2012). Orchid species sampled were *Orchis anthropophora*, *Cephalanthera damasonium* (Julou et al., 2005), *Cephalanthera longifolia* (Pecoraro et al., 2017), *Orchis simia* and *Orchis simia* (Schatz et al., 2010; Lievens et al., 2010), *Orchis tridentata* (Pecoraro et al., 2012), *Orchis militaris* (Shefferson et al., 2008), *Orchis purpurea* (Lievens et al., 2010), *Himantoglossum adriaticum* (Pecoraro et al., 2013), *Limodorum abortivum* (Girlanda et al., 2005), *Spiranthes spiralis* (Duffy et al., 2019), *Ophrys bertolonii* (Pecoraro et al., 2015), *Neottia ovata* (Hans Jacquemyn et al., 2015; Těšitelová et al., 2015), *Neottia cordata* (Těšitelová et al., 2015), *Dactylorhiza baltica* (Shefferson et al., 2008) and *Epipactis atrorubens* (Shefferson et al., 2008).

For each point six variables were extracted by using the ESDAC database (esd) and the WorldClim database (Hijmans et al., 2005): Nitrogen, Potassium and Phosphorus soil content, soil pH, minimum temperature of the coldest quarter and maximum precipitation of the wettest month. Those variables were selected because there is evidence that mycorrhizal fungi are very sensitive to nutrients in the soil: Nitrogen, Phosphorus and Potassium in high quantities (such as in eutrophicated soils because of agricultural fertilizers) have been seen to cause decline in the belowground mycorrhizal fungi species richness and cause dramatic changes in the community composition and structure (Lilleskov et al., 2002; Baar et al., 2002; Grant et al., 2011). Mycorrhizal fungi growth and community composition also seem to be influenced by the soil pH (Aarle et al., 2002; Carrino-Kyker et al., 2016), temperature and precipitation (Rillig et al., 2003). That's not all though: those variables may serve as important proxy for other conditions. Biomes and vegetation are correlated with the environmental condition,

both because they change said conditions (like soil pH) and because all species have a range of tolerance. Also, human impact can often be seen by the amount of chemicals in the soil, especially close to cultivated fields. Environmental values were extracted using GDAL's Sample Raster Values tool (Using QGIS v. 3.16 as a GUI) and appended to the dataset

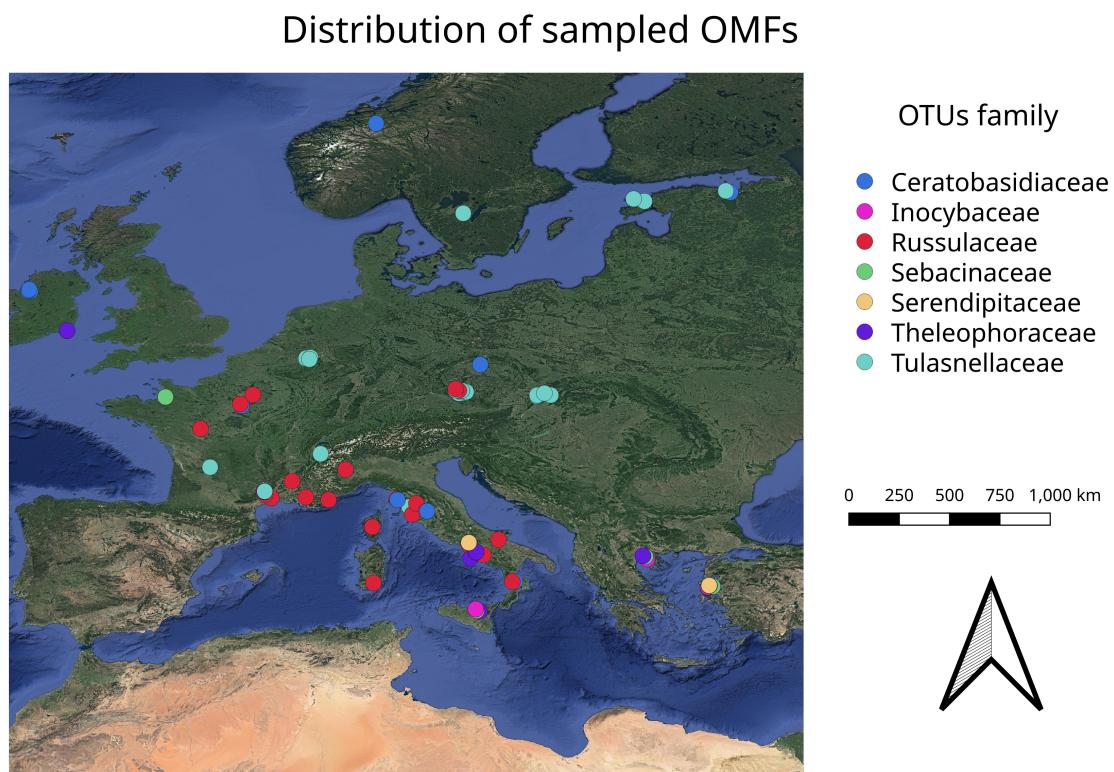


Figure 1.1.: Sampled points

2. Phylogenetic analysis

In order to understand the distribution and ecology of the OrM we need to get a better insight of their phylogenesis. The hypothesis was for sequences of the same family to be clustered together, with some doubts with the Sebacinales as Serendipitaceae and Sebacinaceae are very close and only recently separated (Weiß et al., 2016). The phylogenetic analysis were performed on the sequences deposited by the papers included in the database. The primers used were mainly ITS1F, ITS4, ITS3 and ITS4OF, all targeting regions between the 18S rRNA subunit and the 28S rRNA subunit, including the Internal Transcribed Spacers (ITS hereafter) 1 and ITS 2. Those primers were usually universal for Basidiomycota or in some cases more specific for Tulasnellaceae (like ITS4tul) or other taxa. Sequence DQ520100 from *Tremiscus helvelloides* was used as outgroup.

- Sequences were aligned using the MUSCLE algorithm (Edgar, 2004) and manually trimmed to a visually satisfying overlapping;
- Ugene was used as main GUI, v. 37.0 (Okonechnikov et al., 2012);
- The Maximum Parsimony analysis was performed using TNT, v. 1.1 (tnt), using the Tree Bisection and Reconnection algorithm and with ten replicas. 1000 trees were kept and a strict consensus tree was calculated. A bootstrap was performed on the tree with 200 replications to test the validity of the tree. Bootstrap values are displayed as node labels in the appendix tree;
- the Bayesian analysis (MCMC) was performed using MrBayes, v. 3.2.7a (Huelsenbeck and Ronquist, 2001), using the Hasegawa-Kishino-Yano with a gamma rate heterogeneity among sites (`lset nst=2 rates=gamma;`). One million trees were generated and sampled each thousand, with four chains running. A final consensus tree was then calculated (see appendix).

- Trees were then visually edited with FigTree v. 1.4.4.
- All parameters are available in the supplemental data, along with the files to reproduce the analysis.

3. Multivariate analysis

Before proceeding with the multivariate analysis, sequences have been clustered into Operative Taxonomic Units (OTU hereafter), by using cd-hit v. 4.8.1 (Li et al., 2001). This process yielded 210 OTUs, with the extremes of Serendipitaceae having two OTUs only, and Tulasnellaceae 52 OTUs. The database was then pivoted in a presence-absence matrix, and for further analysis it was splitted by family, so that each matrix only had all the OTUs for that single family, yielding 7 different matrices. This was necessary to test what internal variability each family has; another matrix was obtained by grouping together all the observations from the same family, to test what the variability between the different families is.

A final matrix was obtained by using the single Families/OTUs as rows and removing the orchid species variable. This was done to understand the impact of the environmental variables only on each OTU, therefore trying to understand how different the realized niche (i.e. the variance in the environmental variables) is between the groups.

Principal Component Analysis (PCA hereafter) is an orthogonal linear transformation of the data that aims to maximize the variance of the scalar projection of all points of a dataset into a number of axis ordinated by explained variance. This yields a set of axis, which can be used for clustering, to reduce the number of dimensions and so on. PCA was performed on all matrices using the base R functions `princomp()`, which takes into

3. Multivariate analysis

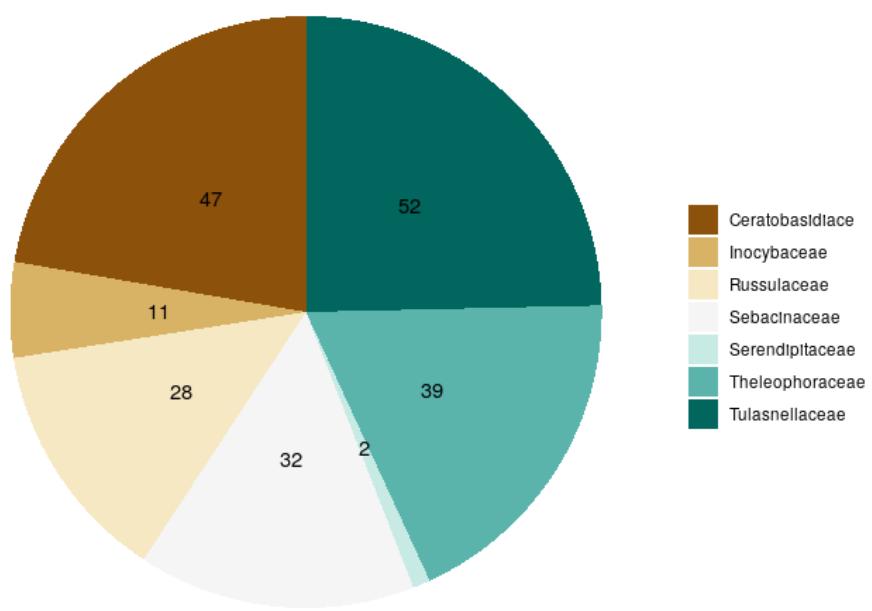


Figure 3.1.: Number of OTUs from each family

3. Multivariate analysis

account the covariance matrix and applies an eigen method of spectral decomposition when possible. Only in the case of Russulaceae, when the number of observation was too limited, `prcomp()` was used, which does a single value decomposition on the centered and scaled data matrix.

As of clustering, Non-metric Multi Dimensional Scaling (NMDS) is another widely used method that allows to visualize the level of similarity of individuals in a dataset. In contrast with PCA, it's non-linear and it's based on a distance matrix, computed by different algorithms depending on the data. It works better with non-parametric data, such as the present one. NMDS was performed on all the matrices by using the R package `vegan` (Dixon, 2003), to understand both how do the OTUs from different families cluster together (if they do) and what environmental factors are most relevant; the Euclidean distance method was used.

In both the PCA and the NMDS we have taken into account how each OTUs presence was influenced by environmental factors, such as climate and soil conditions, and how do they cluster together. Species Distribution Models (SDM hereafter) is another conceptual framework we can use to disentangle the assembly processes that lead to the community as we can observe from the data we have, and to infer the relative importance of the environmental factors. SDMs are numerical tools that combine observations of species occurrence or abundance with environmental estimates. They are used to gain ecological and evolutionary insights and to predict distributions across landscapes, sometimes requiring extrapolation in space and time (Elith and Leathwick, 2009); those models can also inform us of how species-to-species associations depend on the environmental context, in a Joint Species Distribution Model which oftentimes outperforms simple SDMs especially with sparse data (Pollock et al., 2014; Tikhonov et al., 2017).

In the present work, a kind of JSDM called Hierarchical Model of Species Community (HMSC) (Ovaskainen et al., 2017) was performed by using the `Hmsc` package in R, v. 3.0.9 (Tikhonov et al., 2020; `hmsc-r`, 2021); this method uses a bayesian framework to

3. Multivariate analysis

find the best fitting model based on the data, and works very well with presence-absence data as well as with environmental data (Hefley and Hooten, 2016). Three parallel chains were run, sampling every 500 results. Regression was done with a probit model (probability + unit), a non-linear model where the dependent variable can only take two values, which was particularly apt for this dataset because of the binary nature of the presence-absence matrix. Using this framework, a plot with the species responses to environmental covariates (beta parameters) was produced, with at least a 85% posterior probability of being positive (red) or negative (blue). In addition, by using the presence-absence matrix, a correlation plot between the OTUs was established, looking at the positive associations with a statistical support of at least 85% shown in red and negative associations shown in blue.

Part III.

Results

4. Phylogenetic analysis

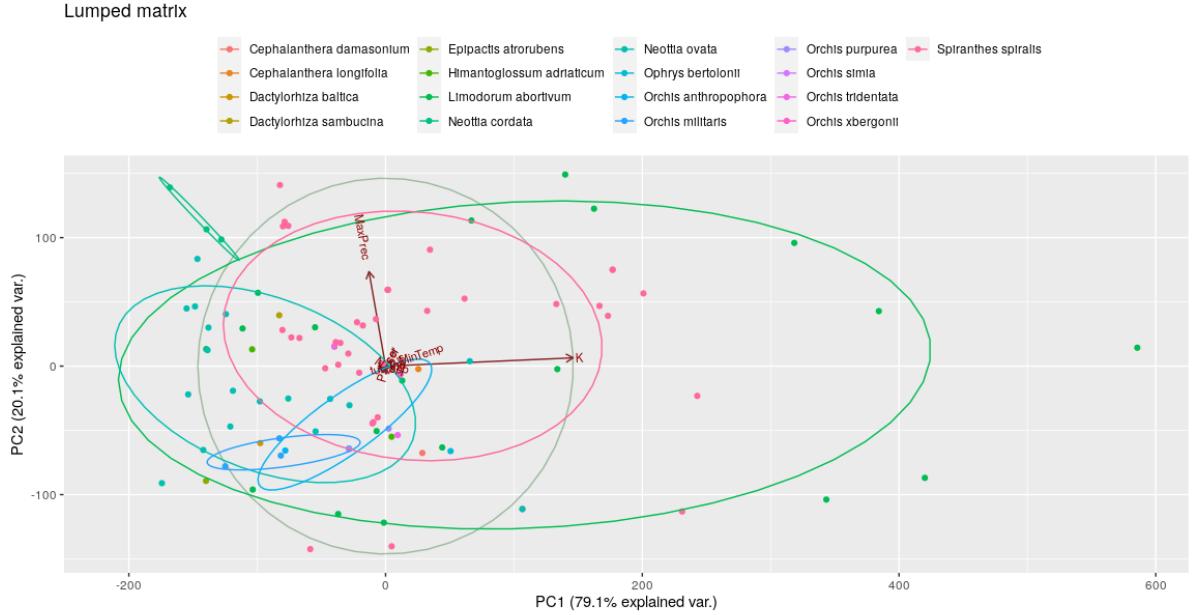
The Bayesian analysis yielded low probability branches. Nonetheless, it correctly put together the families, with the only notable exception of the Serendipitaceae and Sebacinaceae which were nested separately. This makes sense though, as they are both Sebacinales and the Serendipitaceae were originally considered Ssebacinaceae B (Weiss et al., 2004) and were only recently given a new name and properly defined (Weiβ et al., 2016). The Maximum parsimony analysis gave similar results, with very low bootstrap support.

5. PCA

The PCA analysis on the presence-absence matrix and the environmental variables combined showed how there is a substantial overlap of realized niche in the OTUs isolated from different orchids, without distinguishable clusters except for the Tulasnellaceae isolated from *Neottia cordata*. In all cases, the variance was well explained by the first two components (>95% explained variance), with two variables bearing most of the loading: Maximum Precipitation of the wettest month and Potassium content in the soil.

The PCA done using the condensed family matrix yielded the same results, with the

notable exception of the *Limodorum abortivum*, which showed a variance higher than ex-



pected

6. NMDS

The NMDS analysis on the single families seems to show that there is no highly relevant differentiation in the OTUs found in different orchid species, as the clustering wasn't really neat. Again, Tulasnellaceae seem to be the exception, with more distinct groups for different orchid hosts; while this could be a bias caused by the higher number of sam-

ples, Ceratobasidiaceae and Theleophoraceae did not show this pattern even though the sample amount where roughly similar. This could point to a higher specialization of the Tulasnellaceae group, confirming previous observations (Dearnaley, 2007). The NMDS comparing the families yielded only a partial overlapping clustering, which could indicate that different orchids may have different degrees of specialization and realized niche; *Limodorum abortivum* seemed to exhibit the highest diversity, together with *Spiranthes spiralis*.

Taking the Orchid species out of the NMDS analysis and only looking at how different OrM families clustered based on the environmental conditions showed an unexpected pattern. Russulaceae seemed to have a high variance, which points to a broader realized niche, compared to all other families; Tulasnellaceae, which is the most sampled and abundant OrM in the dataset, had less than half the variance and clustered in an area comparable to Sebacinaceae.

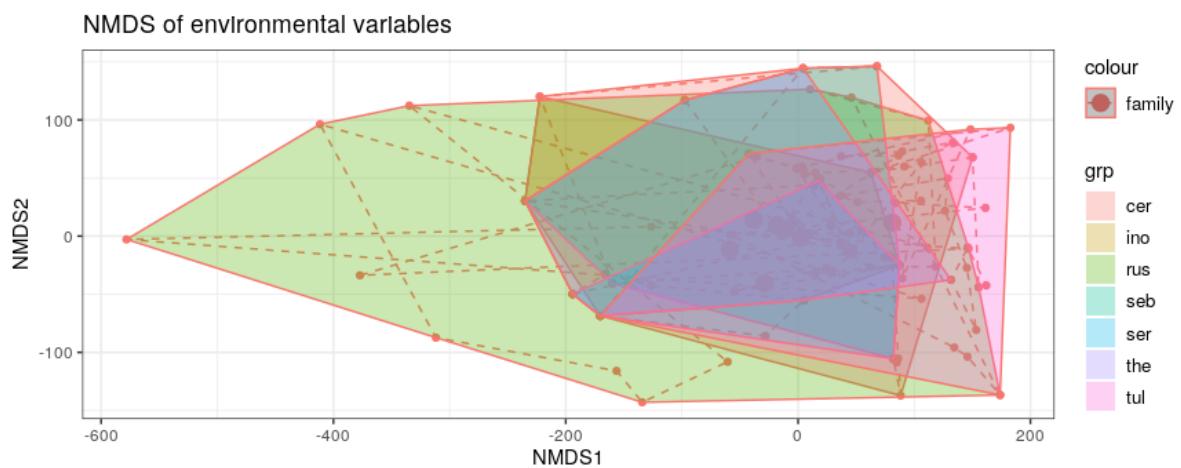


Figure 6.1.: NMDS of the OrMs families considering environmental variables only

7. Hierarchical modelling of species communities

Joint species distribution modelling in HMSC. In the correlation between the families seemed like most families had a positive correlation, with two exceptions: Tulasnellaceae, who had no correlation (0) and Russulaceae, that had a negative correlation (-1). OTUs from the same families seemed, on the other hand, to have no correlation with the others, positive or negative. This stands true for all families but Ceratobasidiaceae, which had more complex correlations, both positive and negative. Whether this is phylogenetically related is to be understood.

The second HMSC result is the correlation between the groups and the environmental variables. The difference between the families wasn't very pronounced, and the most relevant parameter seemed the Minimum Temperature, which was highly correlated with most families (only Tulasnellaceae had 0 correlation), confirming the importance of this environmental parameter in understanding the distribution of OrMs. Maximum precipitation was also invertedly correlated with most families, except for Russulaceae which showed a positive correlation. Of all the soil parameters, pH seemed the most important with a general inverse correlation (the lower the pH, the higher the presence of the OrM). The differences between OTUS from the same families were less clear-cut, showing different correlations for different OTUs in the same family, giving an idea of the diversity that can happen also at low taxonomic levels. It's worthy of notice that Tulasnellaceae showed again the least amount of internal differences, with Ceratobasidiaceae being at

7. Hierarchical modelling of species communities

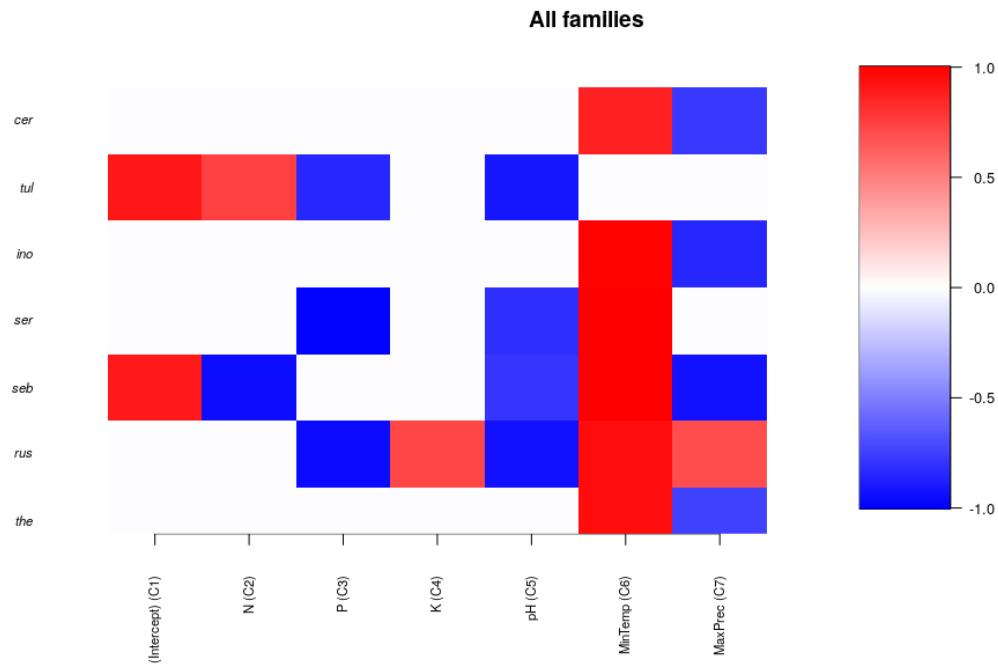


Figure 7.1.: HMSC correlation between the families, taking into account the presence-absence data

the opposite side of the spectrum.

7. Hierarchical modelling of species communities

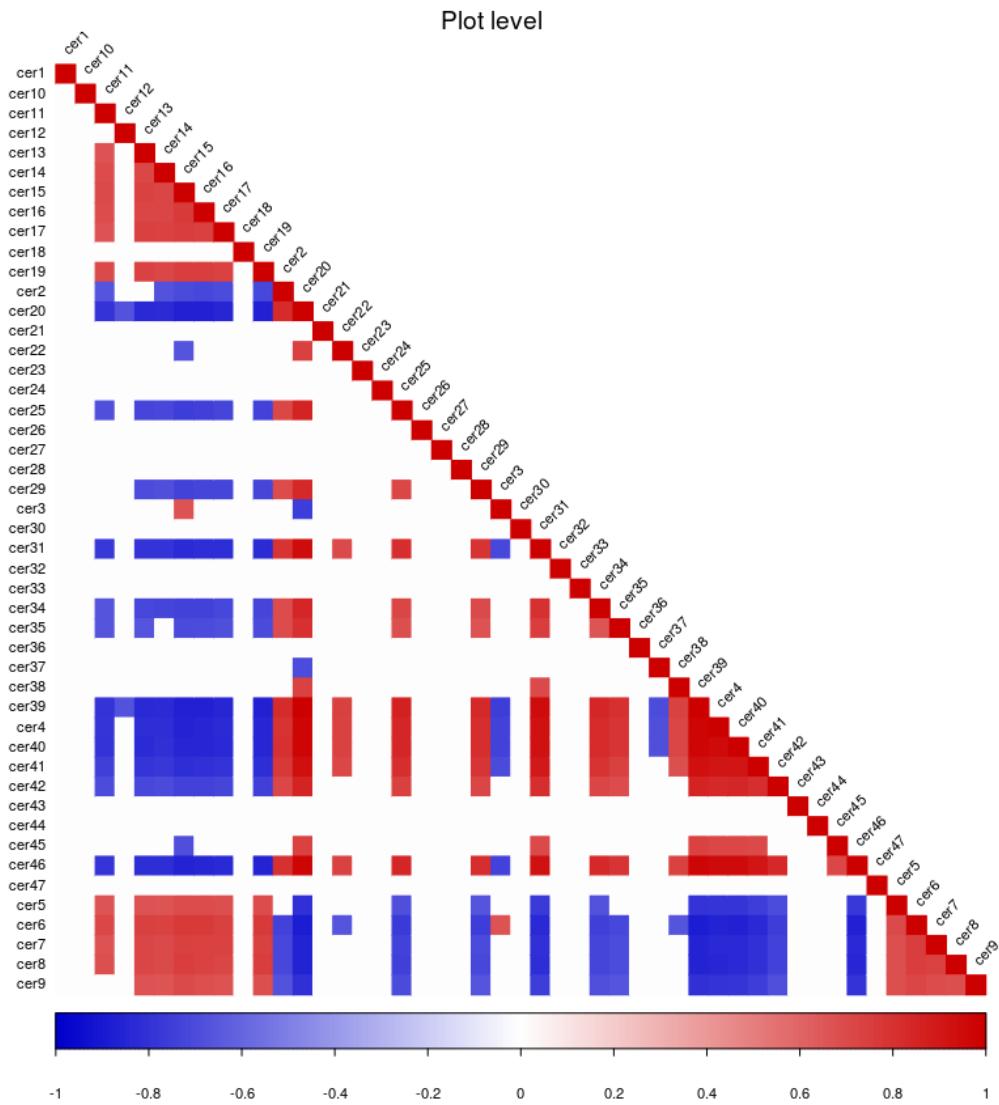


Figure 7.2.: HMSC correlation between the Ceratobasidiaceae OTUs

7. Hierarchical modelling of species communities

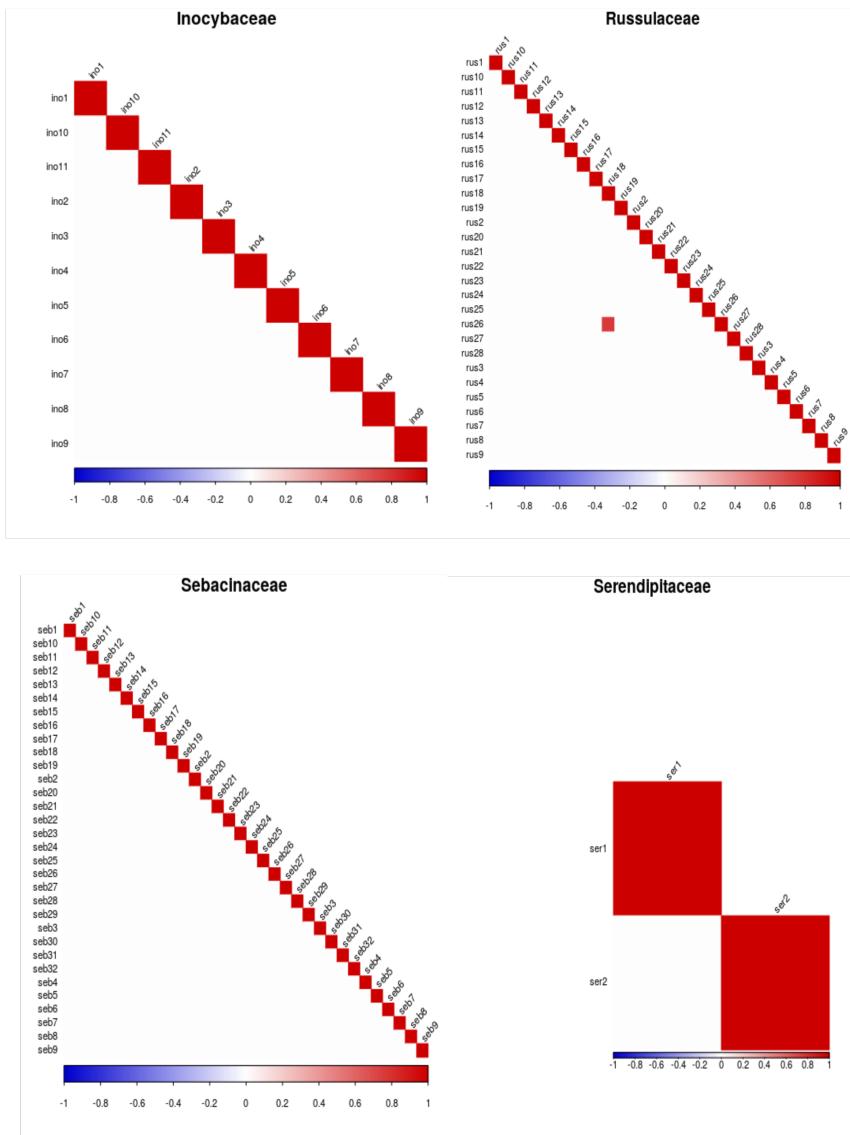


Figure 7.3.: HMSC correlation between the OTUs for Inocybaceae, Russulaceae, Sebacinaceae and Serendipitaceae

7. Hierarchical modelling of species communities

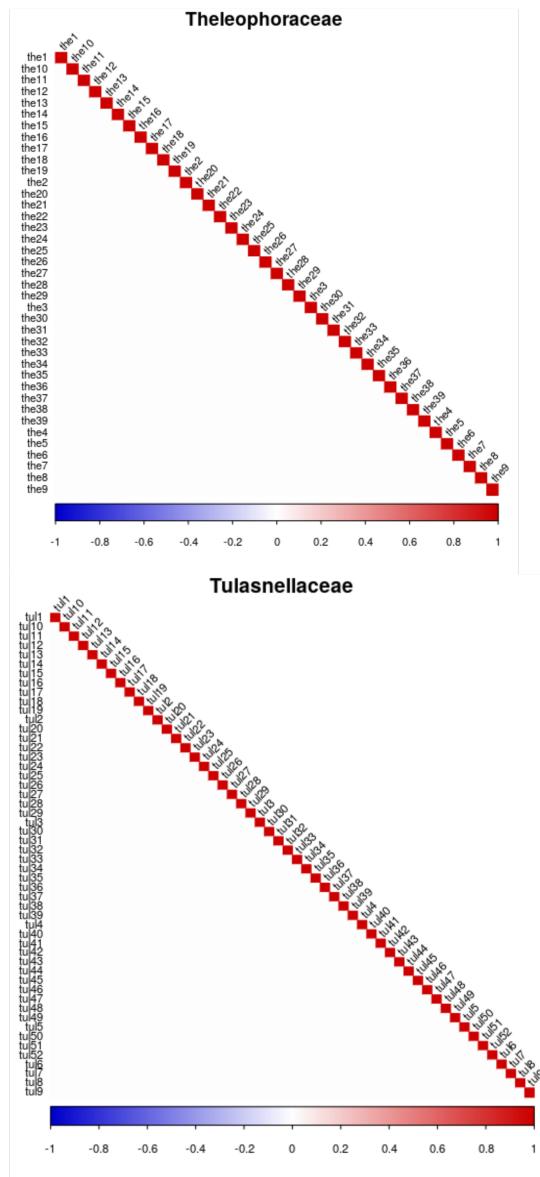


Figure 7.4.: HMSC correlation between the OTUs for Theleophoraceae and Tulasnellaceae

7. Hierarchical modelling of species communities

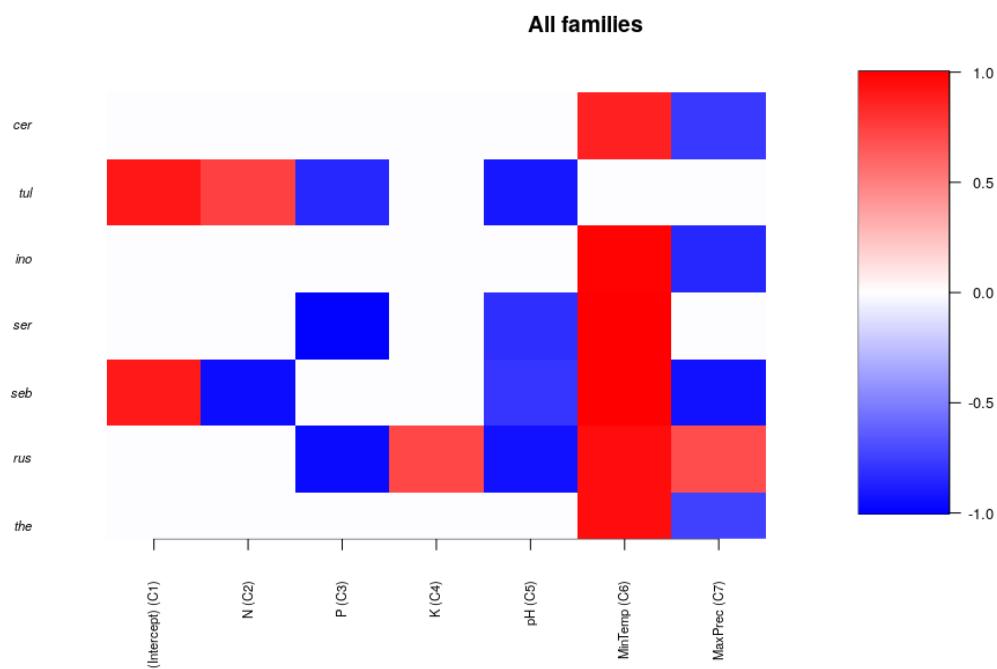


Figure 7.5.: HMSC taking into account the environmental variables for all the families

7. Hierarchical modelling of species communities

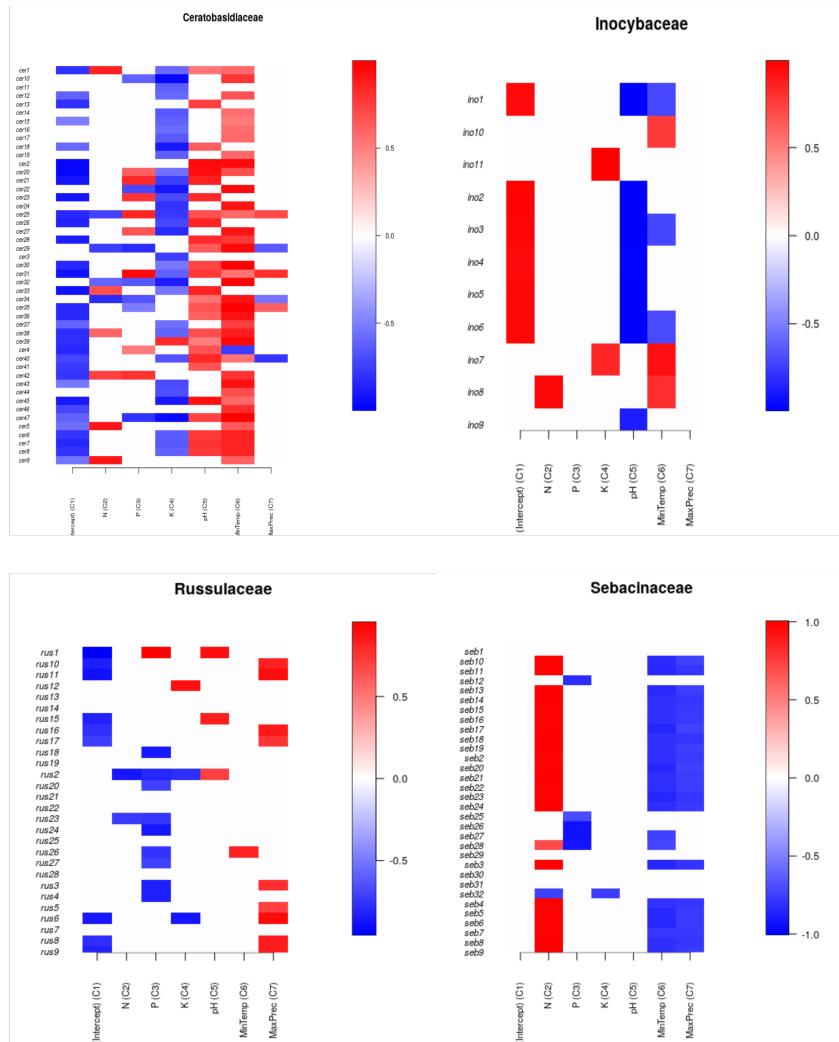


Figure 7.6.: HMSC taking into account the environmental variables for Ceratobasidiaceae, Inocybaceae, Russulaceae and Sebacinaceae OTUs

7. Hierarchical modelling of species communities

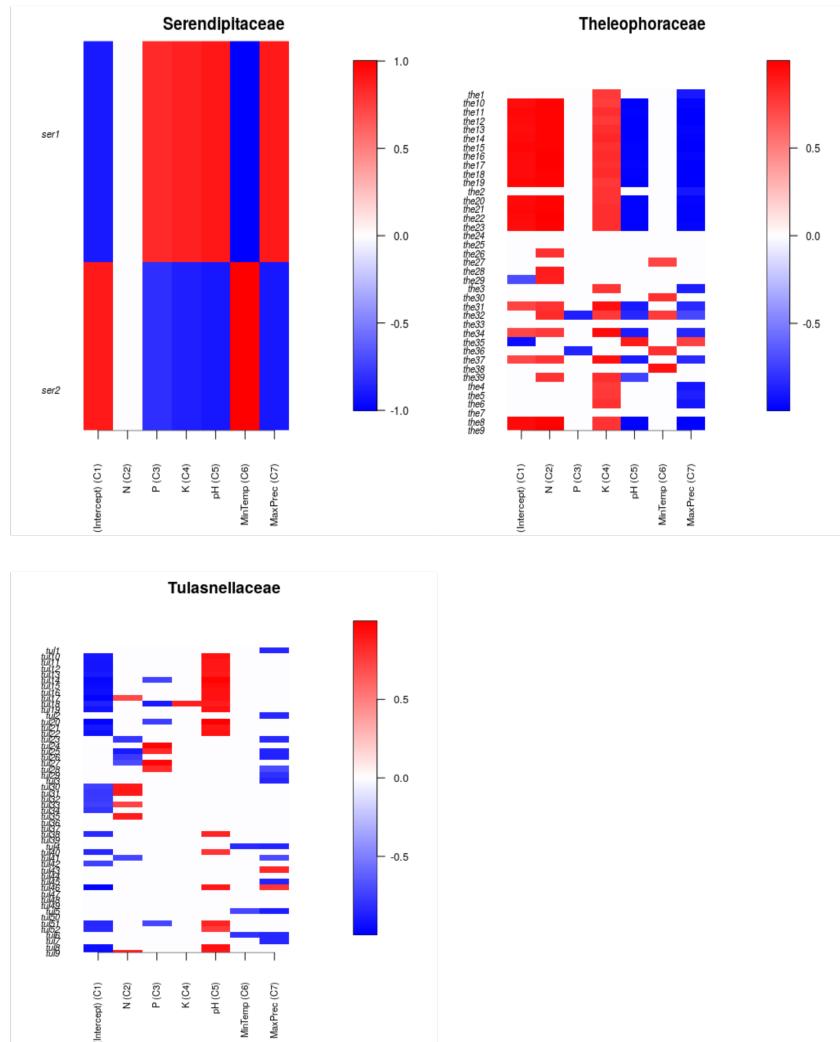


Figure 7.7.: HMSC taking into account the environmental variables for Serendipitaceae, Theleophoraceae and Tulasnellaceae OTUs

Part IV.

Discussion

The PCA analysis was more fruitful. Together with NMDS it seemed to cluster together the fungi isolated from different orchid species, which would support the idea of the OMFs generalist approach as being the most common, which is still an ongoing debate (Bailarote et al., 2012). Also, variance was explained by two main variables, Potassium and Maximum Precipitation, which means that those variables contribute the least to the definition of the niche and that the Orchids were living in places highly differentiated in those parameters. This could also be the result of the very broad sampling, and more research should be done on a smaller scale, as the Potassium levels can dramatically vary even in a very small plot, doubling in just a few meters (Bogunovic et al., 2014).

From the NMDS Russulaceae seemed to be more tolerant than other families to different environments, and despite the lower number of sampled individuals we witness a higher variance. This could mean that the Russulaceae are more generalist toward orchids, that they have a wider niche, that it's more ecologically flexible, or all of the above. Russulaceae are a very diverse family, but it is actually difficult to say that Russulaceae find a “niche” in the symbiosis with orchids, especially with *Limodorum*, as there doesn't seem to be any advantage for the fungus: this orchids seem to have an insufficient photosynthesis and to heavily rely on the OMFs to provide not only minerals but also carbon-base chemicals; this also means that distribution of *Limodorum* may also be potentially constrained by the occurrence of its fungal symbionts (Girlanda et al., 2005).

The HMSC put the spotlight on Russulaceae again, with their negative correlation with the other families. This means that where we find Russulaceae, we are unlikely going to find other OMFs. This is probably the result of the main orchid species where Russulas were found in this dataset, *Limodorum abortivum*, which seems to show a specialization toward this family (Girlanda et al., 2005). The rest of the data seem to confirm a major generalistic approach in the recruitment by orchids, also confirmed by the lack of any correlation between the different OTUs of the same family: the presence of one doesn't seem to inhibit other OTUs from the same family to infect the root.

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