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**The microbial community living within the rust fungus *Uromyces*
hawksworthii assessed through metagenome analysis**

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Abstract

This research aimed to characterize microbial community structure in spores of *Uromyces hawksworthii* taken from aecium and telium phases. The bacterial and fungal communities were characterized based on the 16S RNA and ITS genes, respectively, using the Illumina MiSeq 250bp techniques. Within the bacterial community, the dominant taxonomic groups present in both phases were *Proteobacteria* and *Actinobacteria*, and in the fungal one *Eurotiomycetes*, *Sordariomycetes*, and *Dothideomycetes* were the predominant classes. Despite these overarching dominant taxa in all samples, the relative abundance (RA) and diversity of microbial communities were clearly distinguished according to rust spore phases. *Firmicutes* and *Cyanobacteria* mostly characterized the telium phase, and in aecium ones, *Bacteroidetes* and *Thermi* were most abundant. The bacterial diversity, represented by operational taxonomic units (OTUs), was higher in the aecium phase. For the fungal genes, the *Exobasidiomycetes* and *Pucciniomycetes* were more abundant in the telium phase, and the number of OTUs was higher in the telium phase. These data indicated that the microbial community can be used to distinguish the aecium and telium phases of rust fungi, and this information might explain the difference of abundance of hyperparasites in one phase as compared to the other.

Key words: Aecium, Bacteria, Fungi, Hyperparasite, Metagenome, Telium

Introduction

Rust fungi (Basidiomycota, Pucciniomycetes, Pucciniales) are the most species-rich group of obligate plant-pathogenic fungi. They include many important plant pathogens such as *Puccinia graminis* (Wheat stem rust), *Uromyces appendiculatus* (Common bean rust), *Hemileia vastatrix* (Coffee rust) and *Phakopsora pachyrhizi* (Asian rust of soybean). These fungi are obligate biotrophic parasites with a complex life cycle that often includes two phylogenetically unrelated hosts. They have evolved specialized structures named haustoria, formed within host tissue to efficiently acquire nutrients and suppress host defense responses (Vilela et al., 2015). The genus *Puccinia* has the greatest number of species, and *Uromyces* is the second largest genus of rust fungi (Cummins & Hiratsuka, 2004; Sánchez & Piepenbring, 2014).

Uromyces species parasitize monocots and dicots throughout the world. More *Uromyces* species have been recorded from the Asteraceae, Euphorbiaceae, Fabaceae, Liliaceae, and Poaceae, than for other host families. Among the plant families that include hosts of *Uromyces* is the family Loranthaceae. It comprises more than 900 species of hemiparasitic shrubs. Most species occur in tropical and subtropical areas of Africa, America, Asia, and Europe, where they live on the stems or branches of their host plants (Vidal-Russell & Nickrent, 2008). Ten species of *Uromyces* are known to have Loranthaceae as a host, namely *Uromyces circumscriptus* Neger, *Uromyces euphlebius* Syd. & P. Syd., *Uromyces evastigatus* Cummins, *Uromyces loranthi* H. S. Jacks., *Uromyces nilagiricus* T. S. Ramakr. & K. Ramakr., *Uromyces ornatipes* Arthur, *Uromyces phthirusae* Mayor, *Uromyces socius* Arthur & Holw., *Uromyces urbanianus* Henn., and *Uromyces hawksworthii* Souza, Chaves, Pinho & Dianese (Sánchez & Piepenbring, 2014).

The complete life cycle of rust fungi is usually composed of these stages: stage 0 – pycnium (spermogonia) bearing spermatia (pycniospores), stage I - aecium bearing aeciospores, stage II -

uredinium bearing urediniospores, stage III - telium bearing teliospores, and, stage IV - basidium bearing basidiospores. Considering these stages, rust diseases may be divided into microcyclic, demicyclic and macrocyclic. They can also be autoecious, when all stages occur in just one host plant, or heteroecious if they need an intermediate host to complete their life cycle (Cummins & Hiratsuka, 2004). *Uromyces hawksworthii* is a macrocyclic, autoecious rust fungus, whose aecial stage was described as *Aecidium goyazense* on the same host, *Phthirusa stelis*, in several articles (HENNEN et al. 2005, REZENDE et al. 2015, SOUZA et al. 2015).

There are several kinds of spores produced by rust fungi that use chemical compounds from host plants to start their germination. Normally, during the germination process, hexoses, alcohol dehydrogenase, fructose, sucrose, mannitol and phosphates are produced (V M 2009, 2011). These compounds are also stored in the mother-cells, which function as nutrients for hyperparasitic microorganisms, such as bacteria, actinomycetes and fungi.

More specifically, the hyperparasitic fungi colonize the spores through the germinative pores and tubes. Thus, the rust spores contain a wall membrane to close the pores. The aesciospores are protected within structures composed of peridinal cells (L H 1979). The urediniospores have their own germination pores, which are spread on their surface (R. D, 2003).

Hyperparasitic fungi usually infect the germination pores, where the wall membranes are thinner. This penetration can be visualized by microscopy (Optical and Electronic), where the number and position of germinative pores of aesciospores and urediniospores can be determined (R, 2015).

There are several kinds of hyperparasitic fungi, where the most common genus is *Spharelopsis* spp (*Darluca* spp), which forms scostroms on rust fungi (P, 2005). Additionally, the

cercoporoids germinate together with the uredinia and telia, leaving from stomata of host plants. Some of them can be applied to the biological control of soybean, wheat and coffee rust diseases. The bacteria and actinomycetes are able to colonize the germinative tubes of rust fungi and the natural orifices of host plants (Res 2015).

Previously, the aecium and telium stages of rust from Loranthaceae were distinguished morphologically; however, their sequences had more than 99% of similarity (res 2015). Therefore, hyperparasitism within the rust fungi needs to be investigated, with special attention to distinguishing the aecium and telium phases by their microbial community. We hypothesize that some bacteria and fungi, as well as possessing diversity, are specific according to their phases. This research is focused on the differentiation of the rust fungus phases by evaluating the bacterial and fungal community with metagenome analysis.

Materials and Methods

For these analyses, the material collection and DNA extraction followed previous research (resend et al 2015), and the same genomic DNA was used in the current work. Briefly, the specimens were obtained from living leaves of *Phthirusa stelis* in the Federal District of Brazil. DNA was isolated from three replicates of each phase (aecial and telial) containing frozen spores of *Uromyces hawksworthii* using the DNAeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA concentration was verified by Nanodrop before proceeding to polymerase chain reaction (PCR), and the minimum concentration of $\geq 5\text{ng}/\mu\text{l}$ was kept for further metagenome analysis. The steps from amplicon PCR until the metagenome assay were carried out by Illumina MiSeq at Genome Quebec Co., Canada. The

bacterial 16S rRNA genes were amplified using the primer pairs 534f-CS1 (5'-CCAGCAGCCGCGGTAAT-3') and 783r-CS2 (5'-GGTCTACCMGGGTATCTAATCCKG-3') (Rastogi et al. 2010). And the fungal ITS genes were amplified with the primers ITS1-CS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and 5.8S-CS2 (5'-GGTCTAACTTTYRRCAAYGGATCWCT-3') (Buée et al. 2009). The respective adapters, CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTT) (Fluidigm Co., USA) were used for the primers (Amplicon PCR) and barcodes (Index PCR for library construction). The data were re-assembled and the remaining bioinformatic analysis was performed with the QIIME pipeline (<http://qiime.org>) (Caporaso et al. 2010) applied. The relative abundance of operational taxonomic units (OTUs) and principal component analysis (PCA) on rust fungus spores were also accessed. Non-parametrical tests were used to evaluate the difference between aecial and telial phases in terms of microbial community, before the construction of PCA graphs. The sequences were registered in the DNA Data Bank of Japan (DDBJ) (<http://www.ddbj.nig.ac.jp>).

Results

The total number of sequences obtained from all spore samples were 276,967 (average number of reads - ANR 46,161) and 599,413 (99,902 ANR) for 16S rRNA and ITS rRNA, respectively. The following results were consistently displayed at phylum (16S) and class (ITS) level because the number of reads was considerable, in order to provide the required information at this taxonomic level.

The bacterial relative abundance (RA) within the 16S rRNA profiles at class level indicated that rust spores were mainly colonized by the *Proteobacteria* and *Actinobacteria*. In the aecial phases the RA of *Firmicutes* and *Cyanobacteria* were higher than in telial ones, whereas reciprocity occurred for *Bacterioidetes* and *Thermi* (Fig 1). The OTUs was used to estimate the diversity at species level, where the rarefaction curves indicated that more bacterial OTUs were observed in the aecial than in the telial phase (Fig 2). Principal component analysis (PCA) showed a clear separation between both phases with a significant contrast (Fig 3).

The RA distribution across phases of rust spores indicated that *Dothideomycetes*, *Sordaryomycetes* and *Eurotiomycetes* classes dominated the observed sequences in both phases. The *Dothideomycetes*, *Sordaryomycetes*, *Exobasidiomycetes* and *Pucciniomycetes* were more abundant in telial phases than in aecial ones (Fig 4). A comparison of rarefaction curves illustrating the observed number of fungal OTUs present on rust spore fruit revealed that the highest number of OTUs was obtained in telial phases (Fig 5). Similarly, a PCA analysis of rust spores of fungal communities remarkably distinguished both aecial and telial phases (Fig 6).

The morphological features of both phases are shown in Optical and Scanning Electronic Microscope pictures (Suppl. Fig. 1; Suppl. Fig. 2), obtained after fixing and embedding (Vilela et al., 2015)

Discussion

Many bacteria and fungi have been identified as hyperparasites of rust fungi. Bacteria of the genera *Pseudomonas* (Proteobacteria), *Bacillus* (Firmicutes) and *Arthrobacter* (Actinobacteria) have species reported as hyperparasites of rust fungi. Indeed, species of *Bacillus*, *Pseudomonas* and *Arthrobacter* have been primarily investigated as antagonists against rust fungi,

but few have been evaluated in the field and none have been shown to be effective or practical. Suppression of plant diseases due to the action of endophytic microorganisms has been demonstrated in several pathosystems. Bacterial endophytes are potential biocontrol agents of rust. Several mechanisms may control this suppression, either directly on the pathogen inside the plant by antibiosis and competition for nutrients, or indirectly by induction of plant resistance response (Mishra et al. 2018). There are some successful examples of biological control of rust fungi by bacteria, as follows: (a) yellow rust of wheat (*Puccinia striiformis*) by *Bacillus subtilis* strain QST713 (Reiss & Jørgensen, 2017), and (b) wheat stripe rust (*Puccinia striiformis* f. sp. *tritici*) by an endophytic *Bacillus subtilis* strain E1R-j (Li et al., 2013).

In addition, several fungal genera have also been reported as mycoparasites of rusts, such as: *Fusarium*, *Lecanicillium*, *Simplicillium*, *Trichoderma* and *Verticillium* (Ascomycota, Sordariomycetes), *Ampelomyces*, *Cladosporium*, *Eudarluca* and *Ramularia* (Ascomycota, Dothideomycetes), *Paecilomyces* (Ascomycota, Eurotiomycetes), *Tuberculina* (Basidiomycota, Pucciniomycetes), and *Olpidium* (Chytridiomycota, Chytridiomycetes) (Assante et al. 2004; Berndt, 2017; GAUTHIER, N. W. et al. 2014; James et al. 2016; Lutz et al. 2017). The term ‘fungicolous fungi’ covers species that occur on other fungi as parasites, commensals or saprobionts. A reliable estimate of the number of fungicolous species is not available. Fungicolous fungi include members of all higher taxa of true fungi (Chytridiomycota, Zygomycota, Ascomycota including anamorphic taxa, Basidiomycota) and chromistan fungal analogues (Hyphochytridiomycota, Oomycota). Rust fungi infected by *Eudarluca caricis* and species belonging to *Cladosporium*, *Tuberculina* and *Ramularia* are the most frequently observed fungi hosting other fungi (Nischwitz et al. 2005; Trakunyingcharoen et al. 2014; Tsuneda & Hiratsuka, 1979). There are some successful examples of biological control of rust fungi by fungi, as follows:

(a) leaf rust or brown rust of wheat (*Puccinia recondita*) by *Verticillium lecanii*, *Paecilomyces fumosoroseus*, *Beauveria bassiana*, *Cladosporium cladosporioides* and *Metarrhizium anisopliae* (SHEROZE et al., 2003), and (b) rust (*Uromyces appendiculatus*) of common bean (*Phaseolus vulgaris*) by *Trichoderma harzianum* (Abeyasinghe, 2009).

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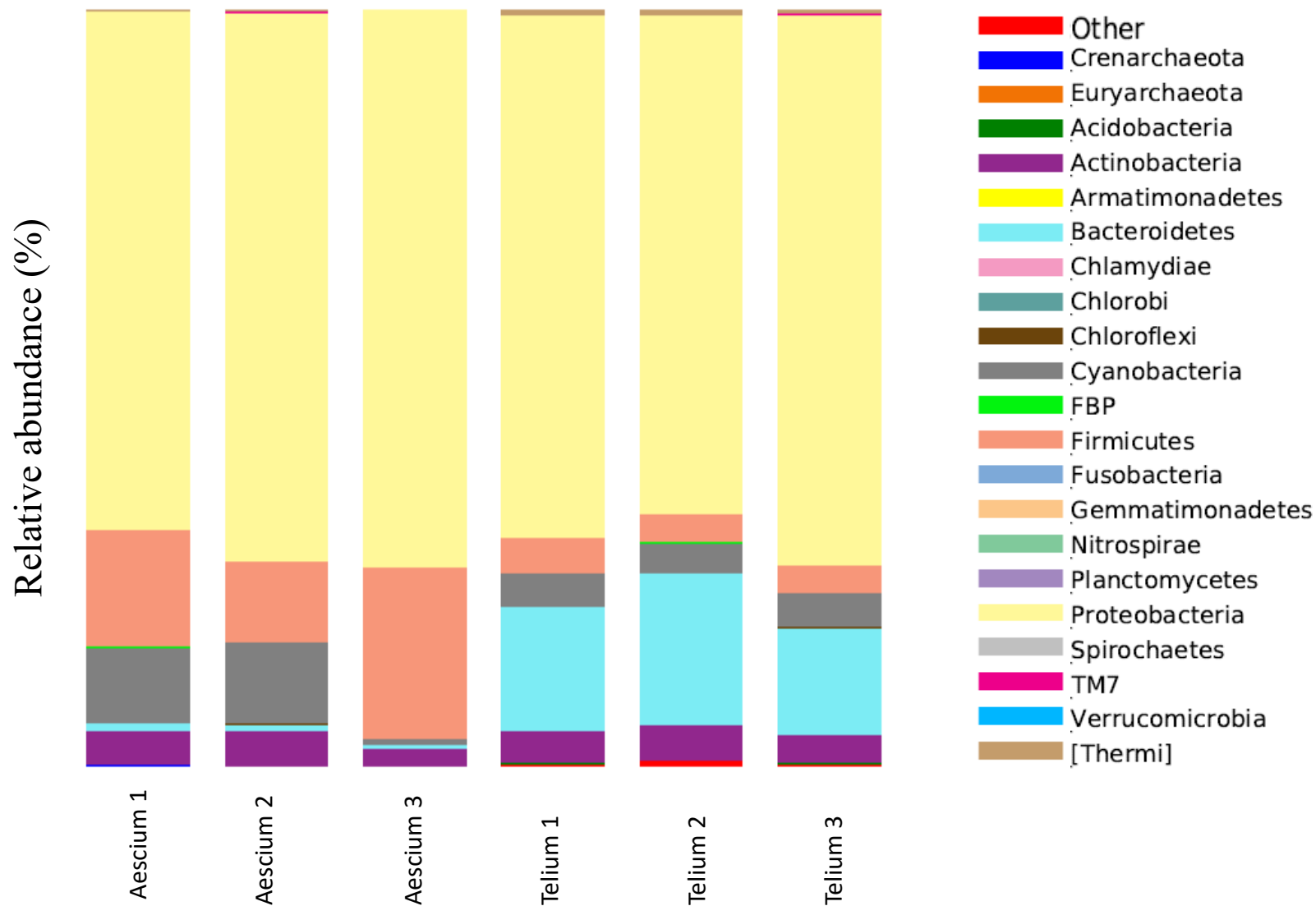


Fig. 1 Distribution of phylogenetical groups of bacteria in rust spores at Aescium and Telium phases

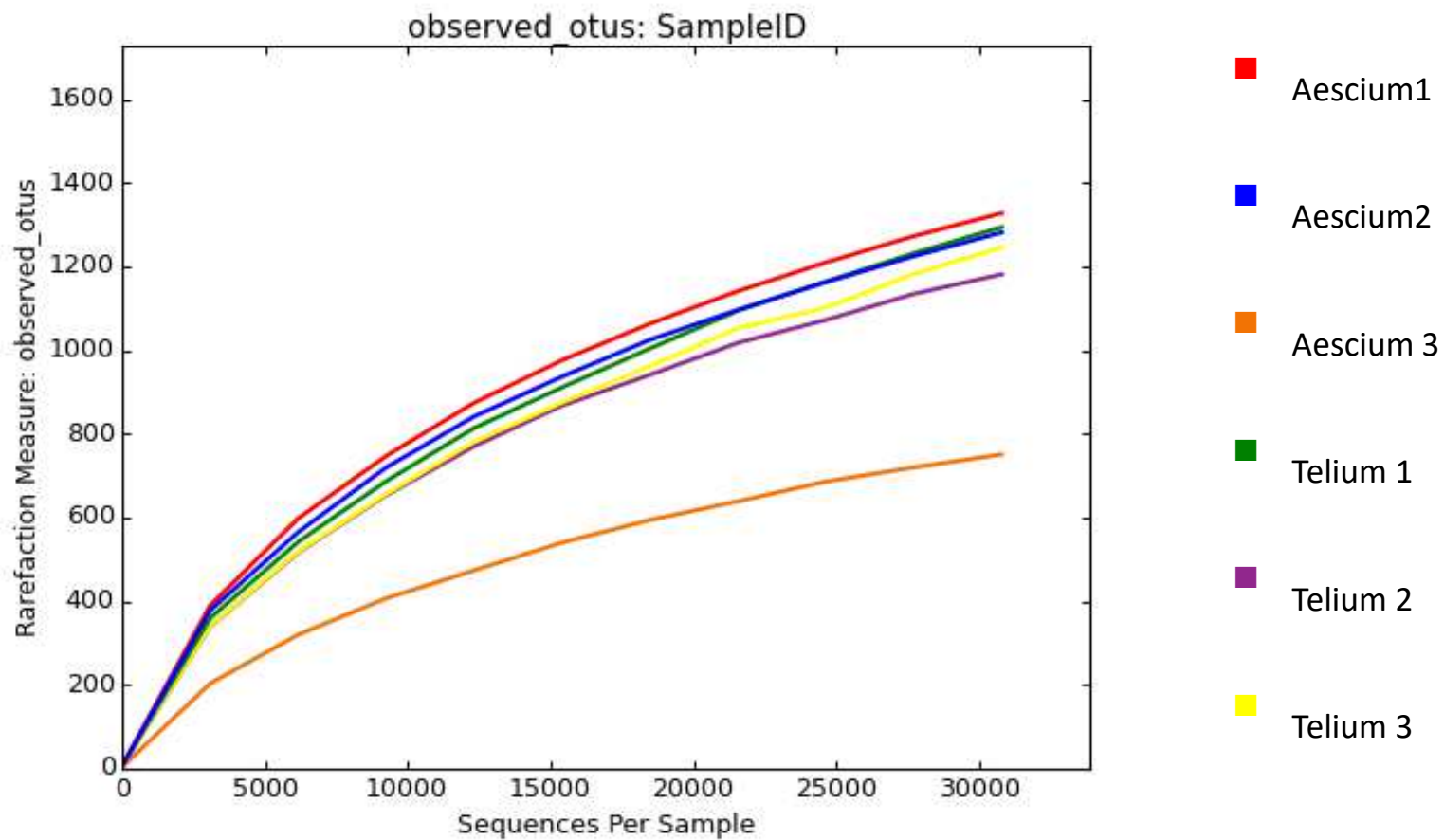


Fig. 2 Rarefactions curves indicating the observed number of OTUs within the 16S RNA gene sequences of the in rust spores at Aescium and Telium phases

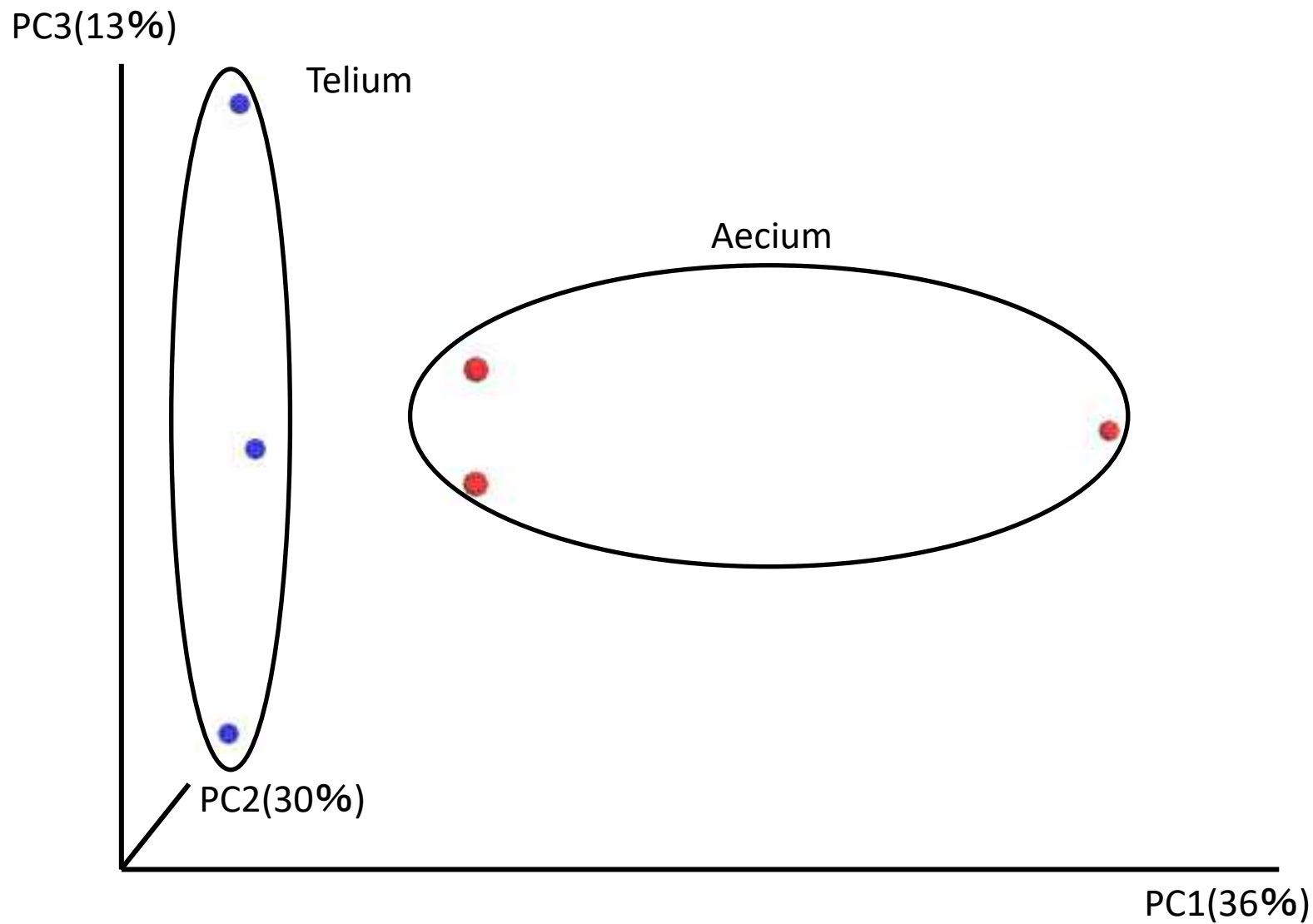


Fig 3. Principal component analyses based on 16S rRNA gene sequences in rust spores at Aescium and Telium phases.

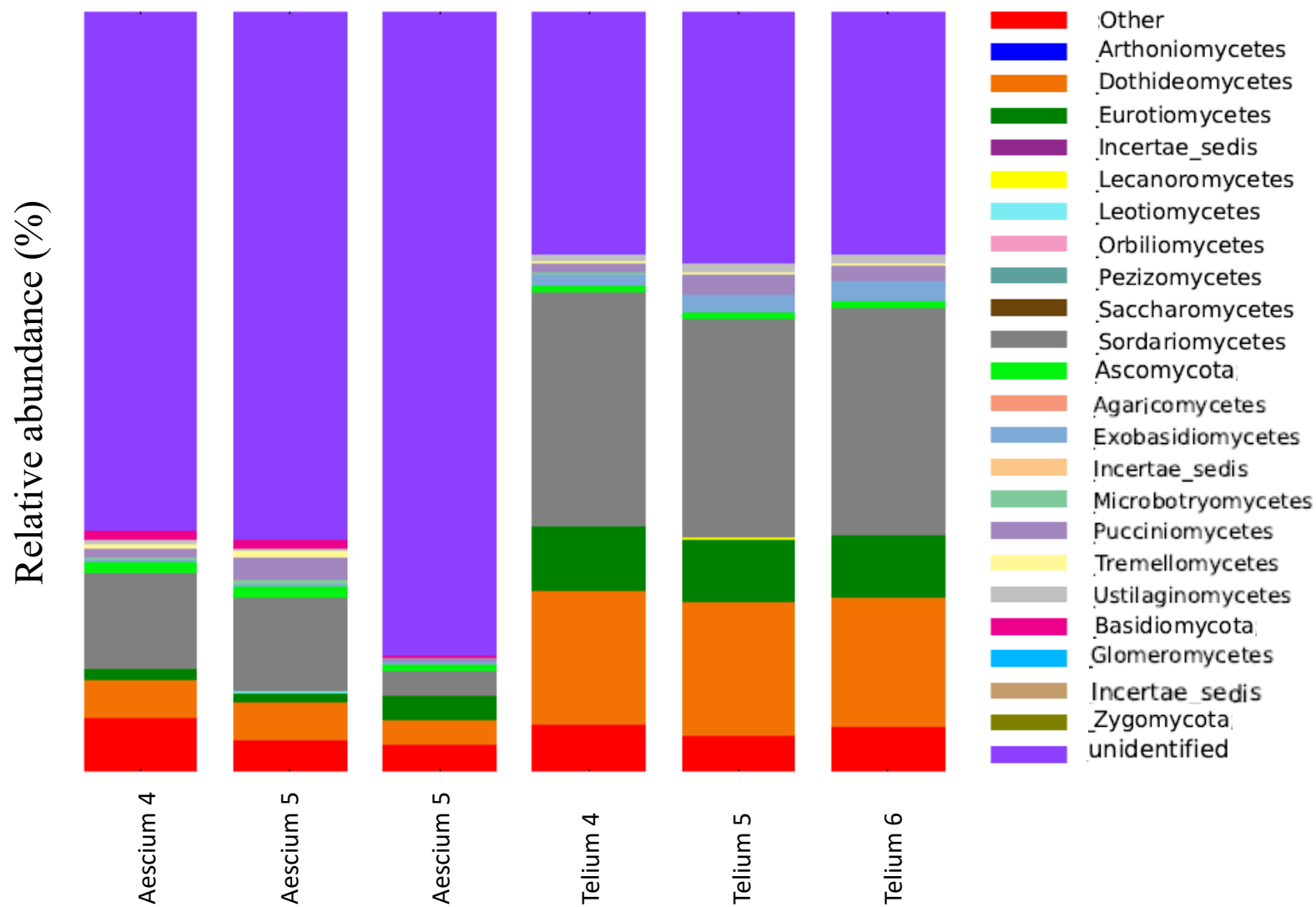


Fig. 4 Distribution of phylogenetical groups of Fungi in rust spores at Aescium and Telium phases.

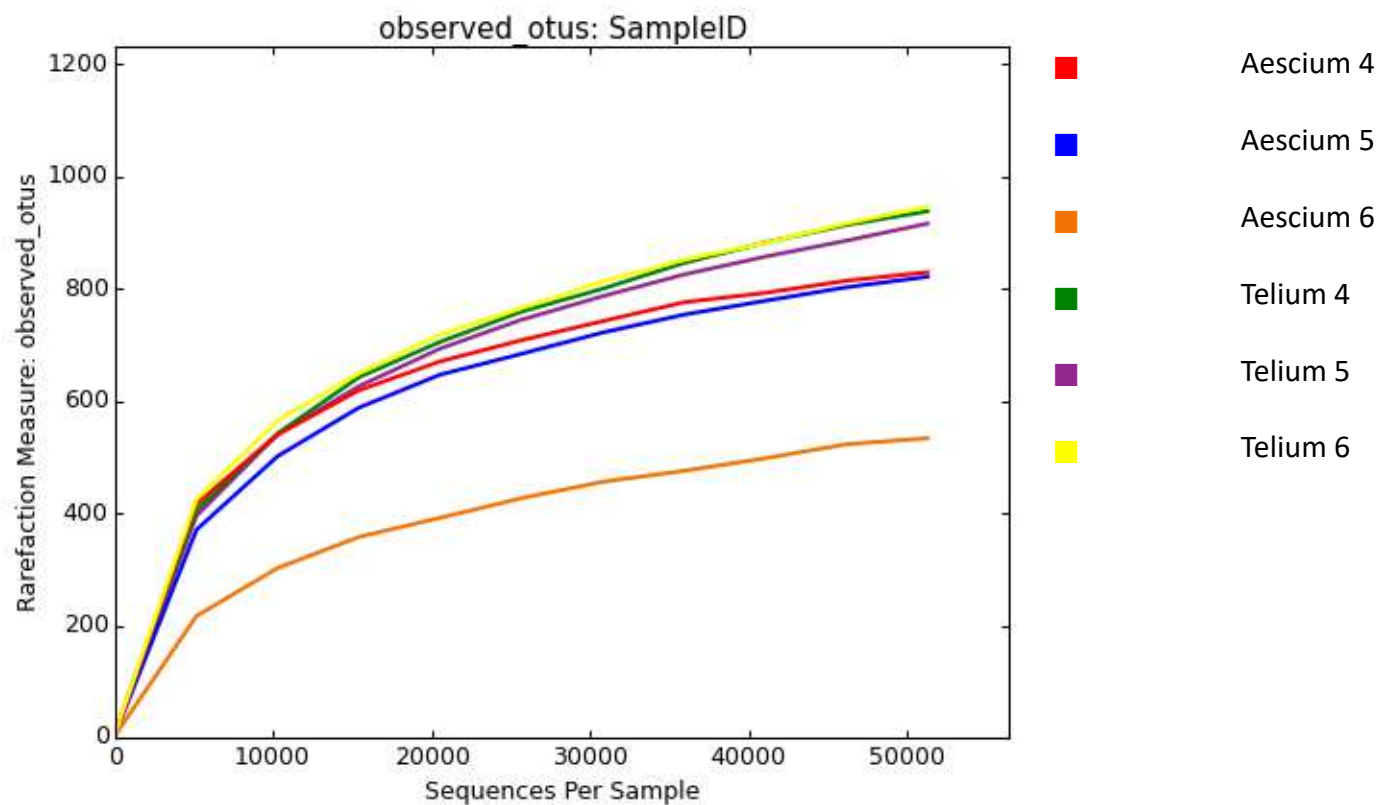


Fig. 5 Rarefactions curves indicating the observed number of OTUs within the ITS rRNA gene sequences in rust spores at Aescium and Telium phases.

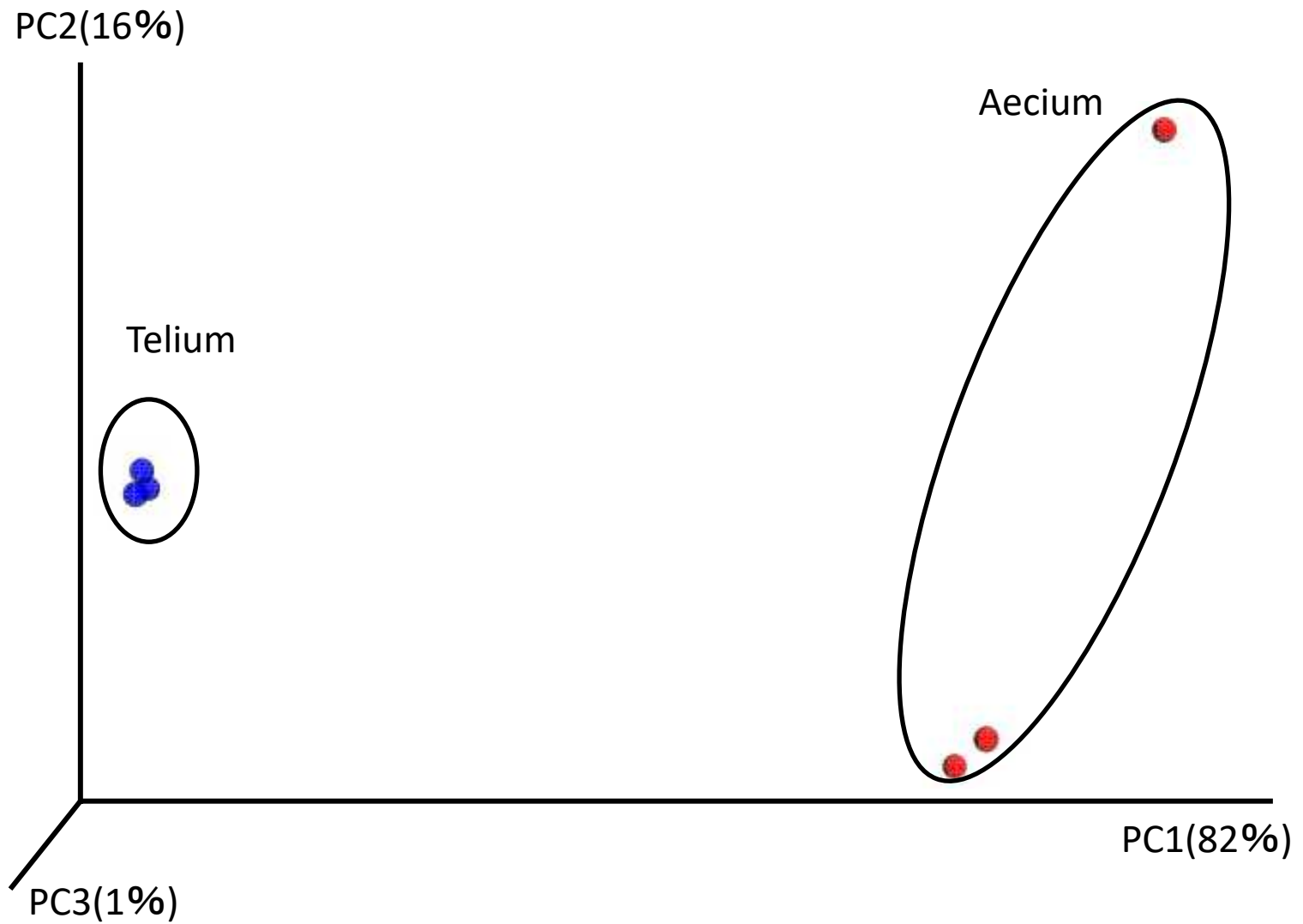
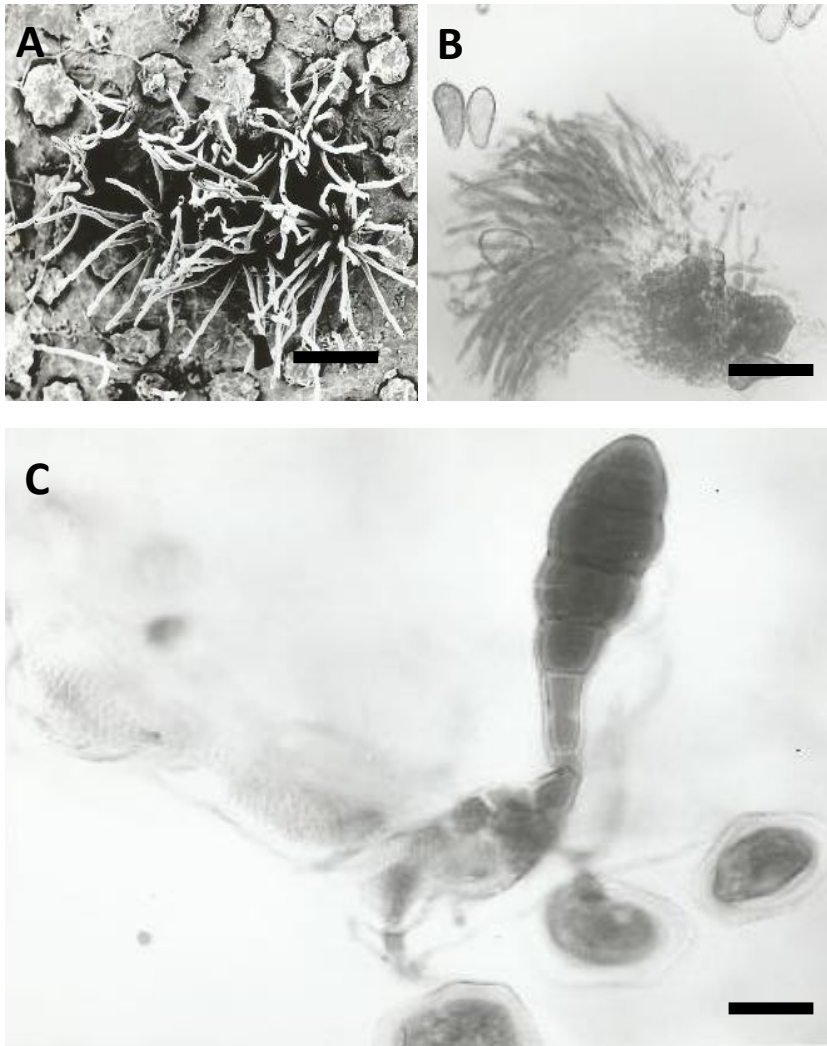
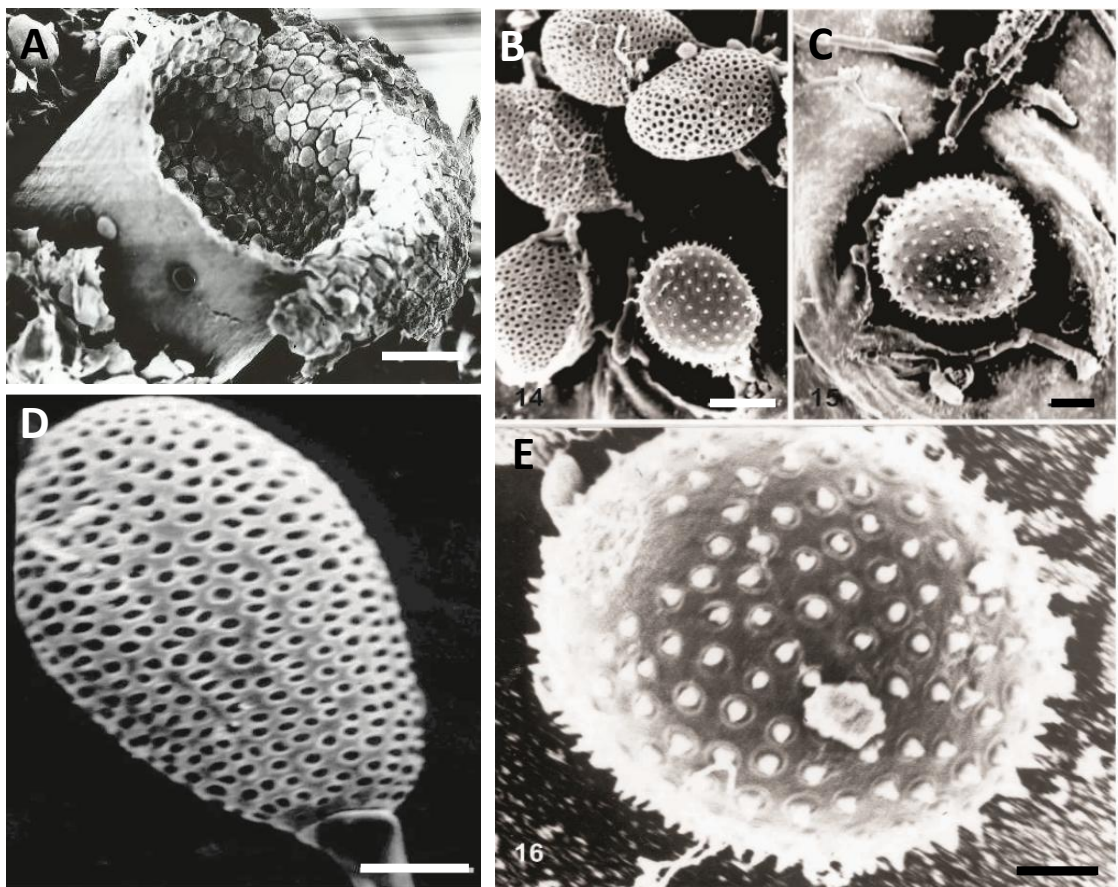


Fig 6. Principal component analyses based on ITS rRNA gene sequences in rust spores at Aescium and Telium phases.



Suppl. Fig. 1 - Uredinia colonized by cercosporoids fungi in Scanning Electronic Microscope (SEM) (A), bar = ... and Optical Microscope (B), bar = ... (C) *Uromyces hawksworthii* aesciospores colonized by *Alternaria* sp., bar = ...



Suppl. Fig. 2 – A - *Uromyces hawksworthii* , Aecidium closed and open with their respective wall cells without hyperparasite colonization. B, C and E- Urediniospores leaving the stomata and some teliospores with light colonization by other fungi hyphae. D – Teliospores with pitted membranes without fungi colonization.