Cladosporium sp. from phyloplane: a diversity evaluation on a Continental ecosystem

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The biodiversity of filamentous fungi on leaves of two genera of plants from a mixed Continental ecosystem (Český Kras, Czech Republic) was assessed. *Quercus* (four species) and *Acer* (three species) are frequent plant genera in the area. Fifteen fungal genera were identified by phenotypic methods (colony characterisation and morphology of reproductive structures). The study focused on *Cladosporium*, the dominant genus, but less common genera were found including *Alternaria*, *Fusarium*, *Rhizopus*, *Penicillium*, *Aspergillus*, *Trichoderma*, and *Absidia*. csM13 fingerprinting and ITS-ARDRA were used for genomic clustering of isolates of *Cladosporium*. Most *Cladosporium* isolates (99%) were identified as *C. herbarum* and *C. macrocarpum*. Diversity indexes and Chi-square analysis were applied to better analyse the fungal community. Continental environments are demonstrated to have some diversity of filamentous fungi, but not as large as in tropical, temperate or Mediterranean areas.

Key words – *Cladosporium* – csM13 PCR "fingerprinting" – Diversity – Fungi – ITS – ARDRA

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Introduction

The phylloplane, or leaf surface, repre sents an important terrestrial habitat that harbours a wide range of microorganisms (Andrews 1991, Lindow & Brandl 2003). Fungi, encompassing both filamentous and yeast taxa, are a major component of the phylloplane microbiota (Dickinson 1976, Andrews 1991, Bills & Polishook 1994, De Jager et al. 2001, Gazis & Chaverri 2010). The leaf surface is a suitable environment for microbial growth because of a thin film of nutrients deposited on the leaf. The fungal communities are influenced by external and/or internal factors such as nutrient availability, humidity, temperature, leaf age and type, and presence of inhibitors (chemical compounds produced by the plant) (Andrews 1991, Fokkema 1991, Kinkel 1997, de Jager et al. 2001, Santamaria & Bayman 2005, Evueh & Ogbebor 2008). Filamentous fungi from the phylloplane may be either parasites, saprophytes, endophytes or epiphytes.

Both parasites and saprophytes can cause disease symptoms or eventually kill the leaf. Endophytes live in leaves causing no visible symptoms and epiphytic fungi exist on the surface of the leaf and apparently causing no damage other than reduction of the photosynthetic potential (Kirk 1995). Some epiphytic fungi protect leaves against infections (Kawamata et al. 2004, van Toor et al. 2005).

Phylloplane populations have been described on many different plant species in temperate (Dickinson 1976, Genilloud et al. 1994), tropical (Bills & Polishook 1994, Kirk 1995, de Jager et al. 2001), Mediterranean (Inácio et al. 2002, Pereira et al. 2002) and Antarctic ecosystems (Möller & Dreyfuss 1996). However, mixed Continental/Oceanic zones have not been fully investigated as natural habitats for microbes in general, and particularly fungi. These ecosystems, which are generally found in the high 40s in latitude, are characterized by very hot summers and very cold winters, with low levels of humidity throughout the year. This type of climate is exclusive to highland locations near Mediterranean climatic areas, such as in Central and Eastern Europe. The particular characteristics of such an ecosystem can be used to study the influence of climate on biodiversity of filamentous fungi, using phylloplane fungi as a bioindicator of diversity. The dominant tree species in these areas are *Quercus petraea*, *Q. robur*, *Carpinus betulus* and *Fagus sylvatica*, while *Acer platanoides* and *Fraxinus excelsior* can also be found (Hofmeister et al. 2002, 2004).

The aim of the present work was to provide information about the abundance and diversity of epiphytic mycobiota in the phylloplane of selected plants growing in a Czech Continental ecosystem (Český Kras), using phenoltypic identification and a molecular approach.

Materials and Methods

Study area

'Český Kras' (49°56′20''N, 14°10′30''E) is a calcareous area with numerous karst phenomena, an altitude between 200 and 500 m, located 25 km SW from Prague (Czech Republic). It is a protected landscape area with communities of forest steppes and broadleaved forests with a very rich flora and fauna. The average annual temperature ranges between 7°C and 8°C, and the average annual precipitation varies between 480 and 560 mm (Hofmeister et al. 2002, Špičková et al. 2008).

Collection of leaves

Leaves (120 per plant species) were collected from two tree genera comprising seven species representative of the Český Kras plant cover: *Acer campestre*, *A. platanoides*, *A. pseudoplatanus*, *Quercus petraea*, *Q. pubescens*, *Q robur* and *Q. rubra*. For each plant species, ten trees more than 50 m apart were chosen. Twelve leaves were collected from distinct parts of each tree. The leaves were picked with sterile forceps and placed in sterile polyethylene bags, which were kept in a cool receptacle until processing (for a period that usually did not exceed 4–5 hours). In a few cases leaves were stored at 4°C for no longer than 24 hours.

Fungi isolation and genus identification

Filamentous fungi were isolated from leaf washings. A preliminary washing step was carried out in order to avoid plating of phyllosphere conidia merely deposited on leaf surfaces (Alhubaishi & Abdel-Kader 1991). Leaves from from each plant species were pooled, in 10 g lots and washed by mechanical shaking in 200 ml of sterilized ultra pure water at room temperature for 1 hour. This was done several times until all the leaves were washed. The water suspension was decanted and and discarded and the process repeated six times. Thereafter, leaves were cut into approximately 10 × 10 mm pieces and 5 g of leaf material from each plant, suspended in 50 ml of sterile Ringer's solution (NaCl 0.45% w/v), was vigorously shaken for 5 min. This procedure, used in previous phylloplane studies (Hogg & Hudson 1966, Mishra & Dickinson 1981, McCormack et al. 1994, de Jager et al. 2001, Inácio et al. 2002), may yield inocula from endophytic mycobiota, but the latter are probably outnumbered by epiphytic cells and should not be significant. Serial dilutions of 10^{-1} to 10^{-4} were spread (0.2 mL in each plate) on 10 Petri dishes containing Cooke Rose Bengal Agar (Oxoid) supplemented with chloramphenicol (0.01% w/v) to prevent bacterial growth.

The plates, incubated in darkness at 25°C, were monitored daily and colonies were counted after 7 to 15 days of growth, according to the amount and speed of growth. At least one colony (isolate) of each macro-morphological type was picked for purification on malt extract agar (MEA) (Merck). Fast growing species were cut out of the plates as soon as they developed and yeasts were not considered during this process. Identification of filamentous fungi followed standard methods based on macro- and micro-morphological features (Raper & Fennel 1965, Samson 1974, Pitt 1979, Domsch et al. 1980).

DNA extraction

Fungal isolates were grown in MEA at 25°C, for 15 to 30 days. Mycelium was scraped from plates with a sterile scalpel, collected into microtubes, and lysed with lysis buffer (50 mM Tris, 250 mM NaCl, 50 mM EDTA, 0.3% SDS (w/v), pH 8,0) mixed with glass beads (425–600 μm) at 60ºC. After centrifugation the supernatant was transferred to clean tubes with Tris-EDTA (10 mM Tris, 1 mM EDTA) and RNAse (Sigma) (60 μg/mL) and incubated for 30 min, at 37°C. Chloroform/isoamyl alcohol (24:1) was then added for extraction. The supernatant was transferred to a new tube and DNA was precipitated with cold ethanol. The resulting pellet was air-dried and resuspended in Tris-EDTA.

csM13 PCR fingerprinting

PCR was performed in 25 μL reaction volumes comprising sterilized ultra pure water, 1 U Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide, 0.2 mM of csM13 primer (5'-GAG GGT GGC GGT TCT-3') (Huey & Hall 1989), 1U Taq DNA polymerase and 250 ng of template DNA, as described by Valério et al. (2005). The DNA was amplified for 40 PCR-cycles with an annealing temperature of 50°C in a Thermo RoboCycler (Stratagene, California) and all reagents were purchased from Life Technologies (England). PCR products were separated electrophoretically through a 1.2% (w/v) agarose gel, at 80 V for 3 h. The gel was stained with ethidium bromide and DNA visualized by UV light.

Amplified ribosomal DNA restriction analysis of internal transcribed spacers (ITS-ARDRA)

PCR was performed in 50 μL volumes containing sterilized ultra pure water, Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide, 0.2 mM of ITS4 primer (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al. 1990), 0.2 mM of ITS5 primer (5'-GGA AGT AAA AGT CGT AAC AAG G-3') (White et al. 1990), 1U Taq DNA polymerase and 250 ng of template DNA. The mixture was amplified for 35 PCR-cycles with an annealing temperature of 55°C. PCR amplifications and separation of products were otherwise as described for csM13 fingerprinting. The amplifycation of ITS region with primers ITS4 and ITS5 produced an amplicon with approximately 600 bp, corresponding to ITS1 +5.8S+ITS2 region, located between 18S and 23S rRNA subunits. ITS regions are highly variable regions situated between 18S, 5.8S and 28S genes which are well known conserved regions within filamentous fungi. Restriction analysis of ITS products was made using endonucleases *Fok*I and *Mva*I (Biolabs). Digestion products were separated electrophoretically through a 2% agarose gel, at 80 V for 3 h. The gel was stained with ethidium bromide and DNA visualized by UV light.

Reference strains

Seven reference strains were acquired from Colección Española de Cultivos Tipo (CECT): CECT 2110 *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, CECT 2107 *C. cucumerinum* Ellis & Arthur, CECT 2665 *C. herbarum* (Pers.) Link; and CABI Bioscience/International Mycological Institute (CABI/IMI): 381056 (CCMI 1016) *C cladosporioides*, 049630 (CCMI 1013) *C. herbarum*, 049452 (CCMI 1014) *C. macrocarpum* and 049639 (CCMI 1015) *C. sphaerospermum*. These species were those most commonly observed in the phylloplane (Hogg & Hudson 1966, Breeze & Dix 1981, Bills & Polishook 1994, Möller & Dreyfuss 1996, de Jager et al. 2001, Inácio et al. 2002, Pereira et al. 2002).

Data analysis

To calculate reproducibility of the isolates in the csM13 figerprinting, 10% of the *Cladosporium* isolates were randomly selected and analysed in duplicate. The similarity between each pair was determined using a dendrogram (results not shown) and the average value across all pairs (reproducibility) was calculated.

The relationships among strains based in the molecular profiles (characters) were analyzed by hierarchical numerical methods using the software package BioNumerics® version 4.0 (Applied Maths, Kortrijk, Belgium). Similarity was computed with Pearson correlation coefficient and the agglomerative clustering was based on Unweighted Pair Group Method with arithmetic Average (UPGMA). Using this software, the profiles obtained with csM13 fingerprinting and ITS-ARDRA were used to produce a combined dendrogram. In the hierarchical analysis, the isolates were positively identified to species only when they clustered with one of the seven reference strains. Intra-specific and inter-specific diversity was evaluated using the indexes of Simpson (Hunter & Gaston 1988) and Shannon-Wiener (Zar 1996). The former index measures the probability of two not related strains, taken from the tested population, belong to two different types and it is based on the number of types and isolates for each type.

The latter index is an evenness measure, expressing the observed diversity as the proportion of the possible maximum diversity and reflecting the homogeneity/heterogeneity of the population. To obtain Shannon-Wiener and Simpson indexes and Pearson's *Chi-Square* values for the cluster analysis (inter-specific and intra-specific diversity) that allow the validation of genetic variability studies, a cutoff of 75% of similarity for inter-specific diversity and of 87% for intra-specific diversity, must be applied to obtain valid clusters. So, for all isolates, the groups were defined at 75% or 87% similarity based in an integrated approach (Fig. 1) and considered as a different type. Chi-Square analysis of contingency was used to test statistical independence among clusters and sample plants.

Results

Phenotypic identification

Leaf washings for all plant species resulted in 460 identified fungal isolates, of which 315 were filamentous fungi and the remaining were yeasts; *Aureobasidium pullulans* was a dominant member of the yeast population.

The highest number of filamentous fungi isolates was obtained from *Quercus robur* (20% of the isolates), followed by *Q. rubra* and *A. platanoides*, both with 17%, *Q. pubescens* (13%), *Q. petraea* and *A. pseudoplatanus* had 12%, respectively. *Acer campestre* had the lowest number of isolates (9%).

Fifteen genera of fungi were identified (Table 1) with a predominance of *Cladosporium*, *Fusarium*, and *Alternaria* (40%, 18%, 14%, respectively) found on all plant species studied. *Rhizopus*, *Penicillium*, *Aspergillus*, *Trichoderma*, and *Absidia* were also observed (in a combined total of 23%) but only on some plants, mainly *Q. robur*, *Q. rubra* and *A. platanoides*. The remaining 5% of fungal species were distributed among seven genera (0.6–1.6%) occurring mostly in *Q. robur*. In *Q. robur* all 15 genera were found but in *Q. rubra* and *A. platanoides* only 12 genera in each, although all genera occurred across the two species. From each of *Q. pubescens*, *Q. petraea* and *A. pseudoplatanus* only eight genera were isolated, but 12 genera were represented across all three species. In *A. campestre* only seven genera were present (Table 1).

Following microscopic examination some species were identified as *Alternaria alternata* (14% of the isolates), *Cladosporium macrocarpum* (8%), *C. herbarum* (15%) and *Fusarium solani* (12%), with an observed total of 19 different species (Table 2). The host plant with the highest number of different species was *Quercus robur* (19) and with the fewest number was *Acer campestre* (11).

Since *Cladosporium* was the dominant genus across the whole sample a more extensive analysis of these isolates was performed using molecular methods. The isolates distribution by plant genera was 45 from *Acer* and 68 from *Quercus*.

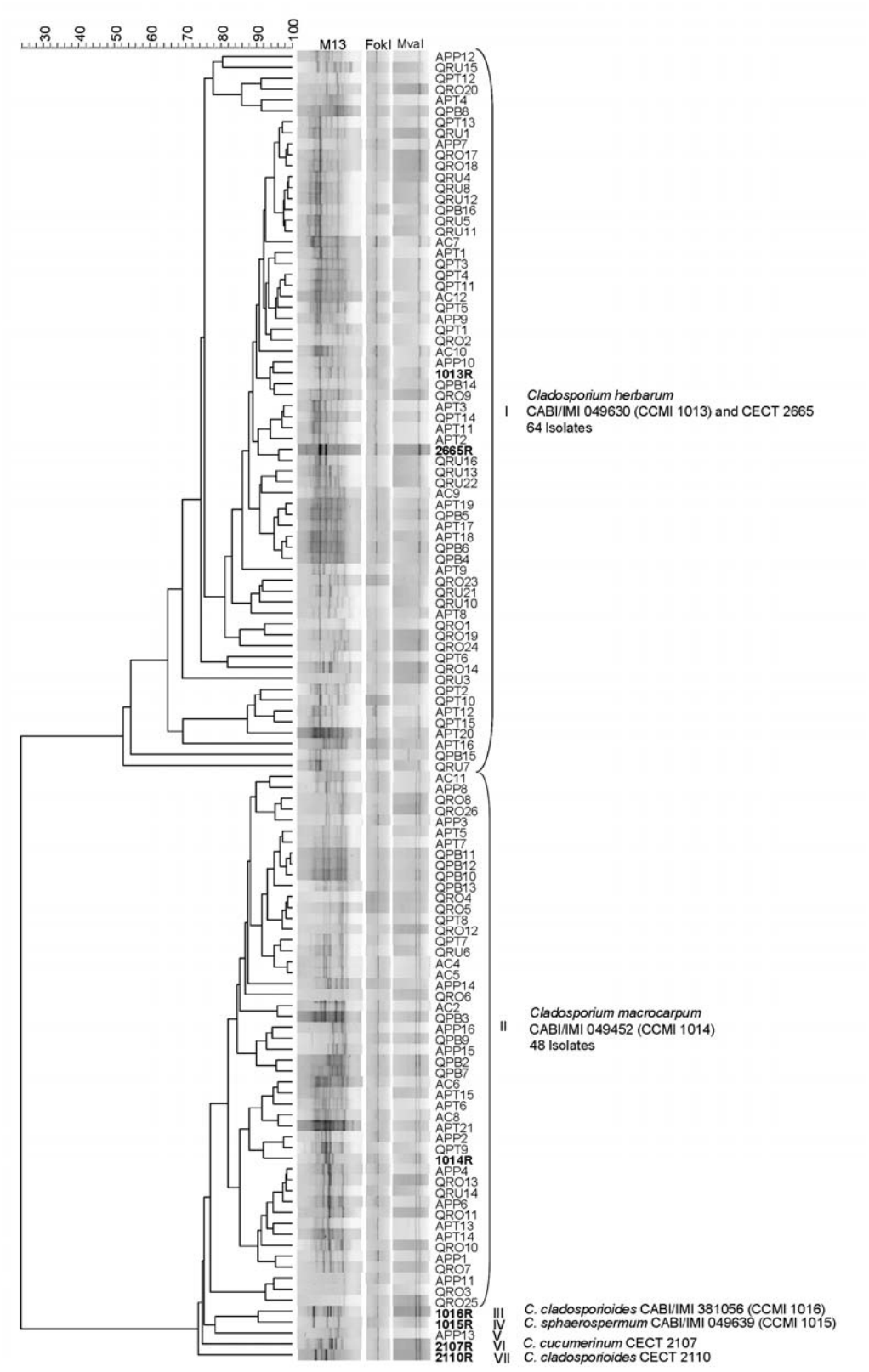

Fig. 1 – Integrative analysis using csM13 fingerprints and ITS-ARDRA.The dendrogram was obtained with Pearson correlation coefficient and UPGMA. The letters preceeding each isolate number indicates the plant species (AC – *Acer campestre*, APP – *A. pseudoplatanus*, APT – *A. platanoides*, QPB – *Quercus pubescens*, QPT – *Q. petraea*, QRO – *Q. robur*, QRU – *Q. rubra*). Isolates with four numbers and an R (in bold) are the reference strains. Roman numbers are used to identify the clusters.

Table 1 Number of colonies of each fungal genus isolated from seven host plant species.

Species \ Plant	*A. campestre*	*A. platanoides*	*A. pseudoplatanus*	*Q. petraea*	*Q. pubescens*	*Q. robur*	*Q. rubra*	**Total**
Cladosporium	15	17	21	15	18	23	18	**127**
Fusarium	5	9	6	8	8	9	11	**56**
Alternaria	5	8	4	7	4	7	9	**44**
Rhizopus	2	3	2	2	3	3	4	**19**
Penicillium	1	3	1	1	2	5	3	**16**
Absidia	1	3	1	2	1	2	2	**12**
Aspergillus	1	2	1	1	2	3	2	**12**
Trichoderma	0	3	0	1	2	4	2	**12**
Trichothecium	0	1	1	0	0	2	1	**5**
Botrytis	0	1	0	0	0	1	0	**2**
Epicoccum	0	1	0	0	0	1	0	**2**
Mucor	0	0	0	0	0	1	1	**2**
Curvularia	0	1	0	0	0	1	0	**2**
Bispora	0	0	0	0	0	1	1	**2**
Bipolaris	0	0	0	0	0	1	1	**2**
Total	**30**	**52**	**37**	**37**	**40**	**64**	**55**	**315**

Table 2 Number of colonies of each fungal species isolated from seven host plant species.

Species \ Plant	*A. campestre*	*A. platanoides*	*A. pseudoplatanus*	*Q. petraea*	*Q. pubescens*	*Q. robur*	*Q. rubra*	**Total**
Cladosporium cladosporioides	4	3	2	5	2	6	1	**23**
C. herbarum	5	6	9	5	7	9	7	**48**
C. macrocarpum	2	3	6	2	3	5	4	**25**
Cladosporium sp.	4	5	4	3	6	3	6	**31**
Fusarium solani	3	4	5	6	3	7	10	**38**
Fusarium sp.	2	5	1	2	5	2	1	**18**
Alternaria alternata	5	8	4	7	4	7	9	**44**
Rhizopus sp.	2	3	2	2	3	3	4	**19**
Penicillium glabrum	1	3	1	1	2	5	3	**16**
Absidia sp.	1	3	1	2	1	2	2	**12**
Aspergillus niger	1	2	1	1	2	3	2	**12**
Trichoderma harzianum	0	3	0	1	2	4	2	**12**
Trichothecium sp.	0	1	1	0	0	2	1	**5**
Botrytis sp.	0	1	0	0	0	1	0	**2**
Epicoccum sp.	0	1	0	0	0	1	0	**2**
Mucor sp.	0	0	0	0	0	1	1	**2**
Curvularia sp.	0	1	0	0	0	1	0	**2**
Bispora sp.	0	0	0	0	0	1	1	**2**
Bipolaris sp.	0	0	0	0	0	1	1	**2**
Total	**30**	**52**	**37**	**37**	**40**	**64**	**55**	**315**

In relation to isolates per plant species the allocation was: *A. campestre* – 10, *A. platanoides* – 15, *A. pseudoplatanus* – 20, *Q. pubescens* – 15, *Q. petraea* – 15, *Q. robur* – 22 and *Q. rubra* – 16.

Cladosporium spp. molecular identification

The application of csM13 primer gave profiles with an average reproducibility value of 97% ± 3. Pearson correlation coefficient and UPGMA showed ten clusters of which only five contained the reference strains to enable positive identification of the isolates. Positive species identification was achieved for 56% of the isolates as *C. cladosporioides*, *C. macrocarpum*, *C. herbarum*, *C. cucumerinum* and *C. sphaerospermum*. The amplification of ITS region with primer pair ITS4/ITS5 produced an amplicon with approximately 600 bp as predicted. ARDRA analysis of the obtained amplicon with *Fok*I showed two profiles, one corresponding to the initial amplicon and the other with two fragments (107 bp and 493 bp). Analysis with *Mva*I produced three different profiles, one corresponding to the initial amplicon, one with two fragments (258 bp and 339 bp) and the other with three (93 bp, 241 bp and 266 bp). The analysis of the restriction profiles allowed the identification of 56 isolates as *C. herbarum*. For the remaining 57 isolates, despite having different restriction profiles, a positive molecular identification was not achieved, because each cluster formed in the dendrogram (results not shown) contained more than one reference strain, not enabling a positive identification.

Data from csM13 fingerprinting and ITS-ARDRA was analysed with Pearson correlation coefficient and UPGMA producing a dendrogram where seven clusters were obtained (Fig. 1). The two major clusters, 99% of the isolates, correspond to *C. herbarum* (57%, cluster I) and *C. macrocarpum* (42%, cluster II), since the respective reference strains are exclusively associated in those clusters. Clusters III and VII correspond to *C. cladosporioides* (CCMI 1016) and *C. cladosporioides* CECT 2110, respectively. *C. sphaerospermum* (CCMI 1015) corresponds to cluster IV and *C. cucumerinum* CECT 2107 to cluster VI. The remaining isolate, APP13, was not positively identified since it could be *C. cladosporioides* or *C. sphaerospermum*.

Most *C. herbarum* isolates were from *Quercus* while *C. macrocarpum* isolates were equally recovered from *Acer* and from *Quercus* (Fig. 2).

Chi-Square analysis of contingency was used to test statistical independence among clusters defined by a composite approach (clusters from Fig. 1) and sampled plants (*Quercus* and *Acer*). No significant association ($p>0.05$) was verified, indicating that no correlation exists between plant genera and fungi species.

Shannon-Wiener (SW) and Simpson (S) indexes were applied to evaluate total isolates inter-specific diversity (at 75% cut level) yielding overall values of 0.52 and 0.60, respectively. The same indexes were used to estimate intra-specific diversity. The estimated values of the indexes for *C. macrocarpum* were 0.82 to SW and 0.76 to S. For *C. herbarum* the value of SW was 0.72 and the value of S was 0.62.

The phenotypic and the molecular identification of the isolates is compared in Table 3. From the 113 *Cladosporium* isolates, 82 were phenotypically identified as *C. macrocarpum*, *C. cladosporioides* and *C herbarum*. From these, 62 isolates kept the same identification by molecular methods, giving a 75.6% congruence of results (Table 3).

Discussion

To evaluate fungal biodiversity in various plants/plant systems, the most common approach is to collect the samples, recover the fungi and analyse them by classical methods: colony characteristics and morphological characteristics such as, conidium size, texture, shape and structure (Hogg & Hudson 1966, Breeze & Dix 1981, Bills & Polishook 1994, Genilloud et al. 1994, Möller & Dreyfuss 1996, Caretta et al. 1999, de Jager et al. 2001, Pepeljnjak & Šegvić 2003, Van Toor et al. 2005). Since species identification without an expertise in mycology is difficult, but nowadays, molecular methods have been used: PCR (Cannon 1997, Sterflinger & Prillinger 2001, Lejon et al. 2005), sequencing (Santa maria & Bayman 2005) or Denaturing Gradien

Gel Electrophoresis (DGGE) and Terminal-Restriction Fragment Length Polymorphism (T-RFLP) (Nikolcheva & Bärlocher 2005).

A Continental plant ecosystem as a model to study the phylloplane fungi of different plant species was assessed by a classical and a molecular approach. The results obtained by classical methods showed several different fungi specie, with the diversity found being intermediate between the Mediterranean (Dickinson 1976, Inácio et al. 2002, Pereira et al. 2002) and the poorer Antarctic (Möller & Dreyfuss 1996) and coastal environments (Genilloud et al. 1994). The closest environment, in terms of number of species and species composition, is the Mediterranean (Inácio et al. 2002, Pereira et al. 2002). However, the full comparison must be taken as approximate because of different isolation methods, number of isolates, and plants.

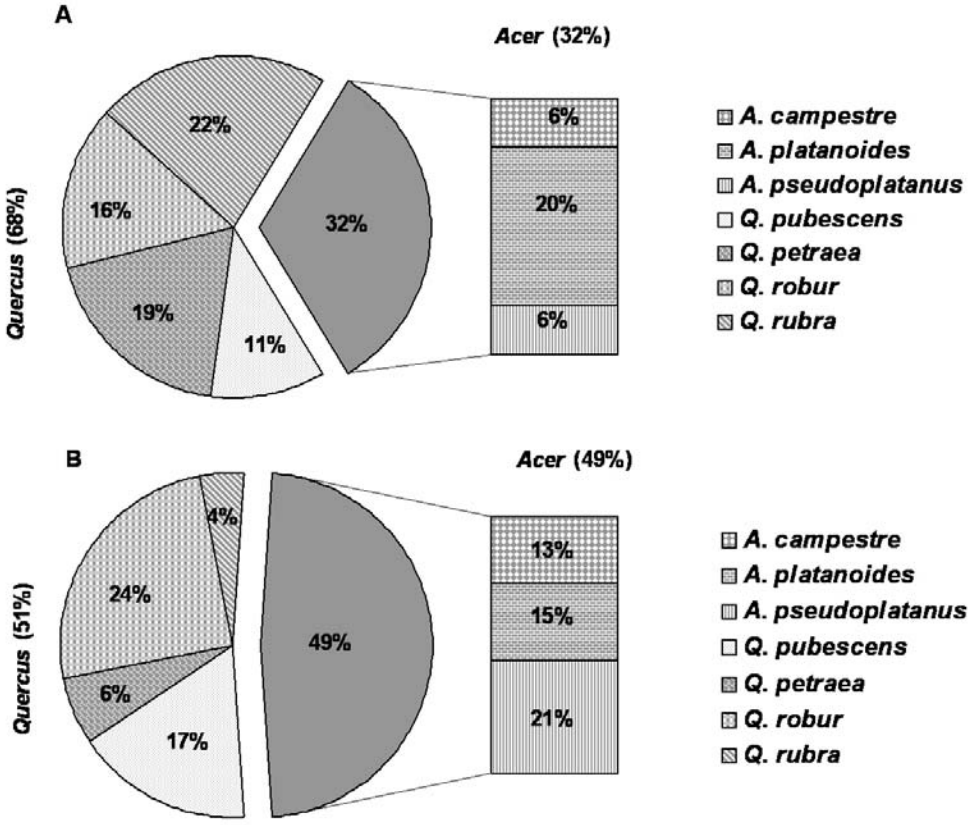

Fig. 2 – Distribution of isolates of *Cladosporium herbarum* **(A)** and *C. macrocarpum* **(B)** by plant species: *Acer campestre*, *A. pseudoplatanus*, *A. platanoides*, *Quercus pubescens*, *Q petraea*, *Q robur*, and *Q. rubra*

Table 3 Phenotypic identification versus molecular identification.

Phenotypic ID. / Molecular ID.	*C. cladosporioides*	*C. herbarum*	*C. macrocarpum*	*Cladosporium* sp.
C. herbarum	9	41		14
C. macrocarpum	10		21	17
C. cladosporioides/ C. sphaerospermum	1			

Phenotypic identification

The highest number of isolates was obtained from *Quercus* suggesting that it is more favourable for the growth and survival of phylloplane filamentous fungi, in this type of climate, than *Acer*. Leaves of *Quercus* are smalller, thicker, with more lobes, than in *Acer*, which could explain the higher number and diversity of isolates in *Quercus*. Among the three *Acer* species, *A. campestre* had a low

number of isolates and represent a low diversity of species.

In most reports of phylloplane fungi there appears to be a marked dominance of anamorphic fungi, mostly of ascomycetous affinity, and the main genera found in the phylloplane are *Cladosporium*, *Aspergillus*, *Alternaria*, *Aureobasidium* and *Epicoccum* (Breeze & Dix 1981, Fokkema 1991, Bills & Polishook 1994, Pereira et al. 2002). Only occasional colonies of basidiomycetous and zygomycetous microfungi have been observed (Hogg & Hudson 1966, Bills & Polishook 1994, Genilloud et al. 1994, Pereira et al. 2002). The three genera more frequently isolated in this work, *Cladosporium*, *Fusarium*, and *Alternaria*, have been described as prominent in phylloplane samples (Dickinson 1976, Breeze & Dix 1981, Caretta et al. 1999, Andrews & Harris 2000, de Jager et al. 2001, Pereira et al. 2002). The genera with moderate occurrence, *Rhizopus*, *Penicillium*, *Aspergillus*, *Trichoderma*, and *Absidia*, have been also reported as being prevalent in the phylloplane (Bills & Polishook 1994, de Jager et al. 2001, Pereira et al. 2002). For the identified species, *Alternaria alternata*, *Cladosporium macrocarpum*, *C. herbarum* and *Fusarium solani*, the dominance is similar to that described by other authors (Dickinson 1976, Breeze & Dix 1981, Fokkema 1991, Andrews & Harris 2000, Pepeljnjak & Šegvić 2003).

Cladosporium spp. molecular identification

As detected in a Mediterranean ecosystem using similar plants (Inácio et al. 2002, Pereira et al. 2002), the most commonly represented genus was *Cladosporium*. Therefore, a more extensive analysis using molecular methods was made with high reproducibility values allowing evaluation of isolate diversity. Usually csM13 fingerprinting is used for strain differentiation. In fact, the cluster analyses of this data for species identification, with the reference strains, allow some reallocation of the isolates, since well defined and separate clusters were obtained for each reference strain. The assessment of ITS-ARDRA is a valuable tool to analyse fungi isolates since ITS is a variable region of the ribosome (White et al. 1990). Any alteration can be easily detected when specific enzymes are used. The restriction enzymes applied in this work were chosen based on *Cladosporium* ITS sequences retrieved from NCBI (AF177734, AF177736) (Sterflinger et al. 2001). Cluster analysis of the restriction fragments permitted one species allocation since it was possible to identify *Cladosporium herbarum* isolates. These isolates were also the easiest to identify by macroscopic and microscopic analysis since they have very specific characteristics such as a smooth velvet look to the colonies and rugous hyphae. The other species are phenotypically similar and even with a molecular approach it was not possible to identify them.

Data from csM13 fingerprinting and ITS-ARDRA was combined and analysed in an integrated approach. Some of the isolates that were not possible to recognize either by microscopic analysis, csM13 fingerprinting or ITS-ARDRA, separately, were identified by the combined molecular methods as *C. macrocarpum*. The identification of *C. herbarum* isolates was confirmed. The two different species positively identified correspond to 99% of the *Cladosporium* isolates. The remaining isolate may belong to *C. cladosporioides* or *C. sphaerospermum*, however, the phenotypic identification obtained for this isolate suggests that the most probable identification is *C. cladosporioides*.

The diversity of *Cladosporium* isolates was tested using Simpson (S) and Shannon-Wiener (SW) indexes. The diversity values showed that the isolates are evenly distributed among the types and enlighten the isolates diversity. These results are similar to those obtained for American and Welsh populations (Fenn et al. 1989, Hunt et al. 2004). The same indexes when applied to the clusters correspondent to each species, also demonstrate that the isolates had diversity. Thus, it is possible to infer that both populations are heterogeneous and the probability of two non related strains belonging to different types is very high. Therefore, these results point to the absence of ecological constraints for both *Cladosporium* species in the maintenance of a high genomic diversity.

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References

Alhubaishi AA, Abdel-Kader MI. 1991 – Phyllosphere and phylloplane fungi of qat in Sana'a, Yemen Arab Republic. Journal of Basic Microbiology 31, 83–89.

Andrews JH. 1991 – Future research directions in phyllosphere ecology. In: Microbial ecology of leave (eds. JH Andrews, SS Hirano, S Verlag). New York, 467–479.

Andrews JH, Harris RF. 2000 – The ecology and biogeography of microorganisms on plant surfaces. Annual Review of Phytopathology 38, 145–180.

Bills GF, Polishook JD.1994 – Abundance and diversity of microfungi in leaf litter of a lowland rain forest rain forest in Costa Rica. Mycologia 86, 187–198.

Breeze EM, Dix NJ. 1981 – Seasonal analysis of the fungal community on *Acer platanoides* leaves. Transactions of the British Mycological Society 77, 321–328.

Cannon PF. 1997 – Strategies for rapid assessment of fungal diversity. Biodiversity and Conservation 6, 669–680.

Caretta G, Piontelli E, Picco AM, Del Frate G. 1999 – Some filamentous fungi on grassland vegetation from Kenya. Mycopathologia 145, 155–169.

De Jager ES, Wehner FC, Korsten L. 2001 – Microbial ecology of the mango phylloplane. Microbial Ecology 42, 201–207.

Dickinson CH. 1976 – Fungi on the aerial surfaces of higher plants. In: Microbiology of aerial plant surfaces (eds. TF Preece, CH Dickinson). Academic Press, London, 293–324.

Domsch KH, Gams W, Anderson TH. 1980 – Compendium of soil fungi, Academic Press, London.

Evueh GA, Ogbebor NO. 2008 – Use of phylloplane fungi as biocontrol agent against *Colletotrichum* leaf disease of rubber (*Hevea brasiliensis* Muell. Arg.). African Journal of Biotechnology 7, 2569–2572.

Fenn ME, Dunn PH, Durall DM. 1989 – Effects of ozone and sulphur dioxide on phyllosphere fungi from three tree species. Applied and Environmental Microbiology 55, 412–418.

Fokkema NJ. 1991 – The phyllosphere as an ecologically neglected milieu: a plant pathologist's point of view. In: Microbial ecology of leaves (eds. JH Andrew SS Hirano). Springer, New York, 3–18.

Gazis R, Chaverri P. 2010 – Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. Fungal Ecology 3, 240–254.

Genilloud O, Peláez F, González I, Díez MT. 1994 – Diversity of actinomycetes and fungi on seaweeds from the Iberian coasts. Microbiología, Sociedad Española de Microbiología 10, 413–422.

Hofmeister J, Mihaljevič M, Hošek J, Sádlo J. 2002 – Eutrophication of deciduous forests in the Bohemian Karst (Czech Republic): the role of nitrogen and phosphorus. Forest Ecology and Management 169, 213–230.

Hofmeister J, Mihaljevič M, Hošek J. 2004 – The spread of ash (*Fraximus excelsior*) in some European oak forests: an effect of nitrogen deposition or successional change. Forest Ecology and Management 203, 35–47.

Hogg BM, Hudson HJ. 1966 – Micro-fungi on leaves of *Fagus sylvatica*. I. The microfungal succession. Transactions of the British Mycological Society 49, 185–192.

Huey B, Hall J. 1989 – Hypervariable DNA. Fingerprinting in *Escherichia coli*: minisatellite probe from bacteriophage M13. The Journal of Bacteriology 171, 2528–2532.

Hunt J, Boddy L, Randerson PF, Rogers HJ. 2004 – An evaluation of 18S rDNA approaches for the study of fungal diversity in grassland soils. Microbial Ecology 47, 385–395.

Hunter PR, Gaston MA. 1988 – Numerical index of the discriminatory ability of typing schemes: an application of Simpson's index of diversity. Journal of Clinical Microbiology 26, 2465–2466.

Inácio J, Pereira P, Carvalho M, Fonseca Á, Amaral-Collaço MT, Spencer-Martins I. 2002 – Estimation and diversity of phylloplane mycobiota on selected plants

in a Mediterranean-type ecosystem in Portugal. Microbial Ecology 44, 44–353.

Kawamata H, Narisawa K, Hashiba T. 2004 – Suppression of rice blast by phylloplane fungi isolated from rice plants. Journal of General Plant Pathology 70, 131–138.

Kinkel LL. 1997 – Microbial population dynamics on leaves. Annual Review of Phytopathology 35, 327–347.

Kirk PM. 1995 – Inventorying microfungi on tropical plants. In: Microbial Diversity and Ecosystem Function (eds. D Allsopp, RR Colwell, DL Hawksworth). CAB International, Wallingford, UK, 355–360.

Lejon DPH, Chaussod R, Ranger J, Ranjard L. 2005 – Microbial community structure and density under different tree species in an acid forest soil (Morvan, France). Microbial Ecology 50, 614–625.

Lindow SE, Brandl MT. 2003 – Microbiology of the phyllosphere. Applied Environmental Microbiology 69, 1875–1883.

McCormack PJ, Wildman HG, Jeffries P. 1994 – Production of antibacterial compounds by phylloplane-inhabiting yeasts and yeastlike fungi. Applied and Environmental Microbiology 60, 927–931.

Mishra RR, Dickinson CH. 1981 – Phylloplane and litter fungi of *Ilex aquifolium*. Transactions of the British Mycological Society 77, 329–337.

Möller C, Dreyfuss MM. 1996 – Microfungi from Antarctic lichens, mosses and vascular plants. Mycologia 88, 922–933.

Nikolcheva LG, Bärlocher F. 2005 – Seasonal and substrate preferences of fungi colonizing leaves in streams: traditional versus molecular evidence. Environmental Microbiology 7, 270–280.

Pepeljnjak S, Šegvić M. 2003 – Occurrence of fungi in air and on plants in vegetation of different climatic regions in Croatia. Aerobiologia 19, 11–19.

Pereira PT, Carvalho MM, Gírio FM, Roseiro JC, Amaral-Collaço MT. 2002 – Diversity of microfungi in the phylloplane of plants growing in a Mediterranean ecosystem. Journal of Basic Microbiology 42, 396–407.

Pitt JI. 1979 – The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Academic Press, London and New York

Raper KB, Fennel DI. 1965 – The genus *Aspergillus*. Williams & Wilkins, Baltimore.

Samson RA. 1974 – *Paecilomyces* and some allied hyphomycetes. Studies in Mycology 6, 8-19.

Santamaria J, Bayman P. 2005 – Fungal epiphytes and endophytes of coffee leaves (*Coffea arabica*). Microbial Ecology 50, 1–8.

Špičková J, Dobešová I, Vach M, Skřivan P. 2008 – The influence of the limestone quarry Čertovy schody (Czech Republic) on the precipitation chemistry and atomspheric deposition. Chemie der Erde 68, 105–115.

Sterflinger K, Prillinger H. 2001 – Molecular taxonomy and biodiversity of rock fungal communities in an urban environment (Vienna, Austria). Antonie van Leeuwenhoek 80, 275–286.

Valério EPP, Saker ML, Franc S, Tenreiro R. 2005 – Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters. Harmful Algae 4, 1044–1052.

Van TRF, Pay JM, Jaspers MV, Stewart A. 2005 – Evaluation of phylloplane microorganisms for biological control of camellia flower blight. Australasian Plant Pathology 34, 525–531.

White TJ, Bruns T, Lee S, Taylor J. 1990 – Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: A guide to methods and applications PCR protocols (eds. MA Innis, DH Gelfand, JJ Sninsky, TJ White). Academic Press, San Diego, California.

Zar JH. 1996 – Biostatistical analysis, 3rd edn. Prentice-Hall International, Inc.