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Relationship between genome size, serpentine adaptation and cryptic sexuality in the ectomycorrhizal fungus *Cenococcum geophilum*

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica da Doutora Susana Gonçalves (Universidade de Coimbra) e do Professor Doutor João Loureiro (Universidade de Coimbra)

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Abreviations

ANOVA – Analysis of variance

CV – Coefficient of variation

EC_{50} – Effective concentration of metal that inhibits growth by 50 %

ECM – Ectomycorrhizal

EDTA $Na_2.2H_2O$ – Ethylenediaminetetraacetic acid

et al. – (L. et alia) and other

FCM – Flow cytometry

FL – Fluorescence pulse integral

FS – Forward light scatter

ITS – Internal transcribed spacer

laser – Light amplification by stimulated emission of radiation;

Mbp – Mega basepair

$MgCl_2.6H_2O$ – Magnesium chloride hexahydrate

NaCl - Sodium chloride

PCR – Polymerase chain reaction

PDA – Potato dextrose agar

pg – Picograms;

Taq – *Thermus aquaticus* (polymerase)

TI – Tolerance index

Tris-HCl – Tris(hydroxymethyl)aminomethane

SE – Standard error

SS – Side light scatter

Resumo

Os fungos micorrízicos constituem um grupo diverso de microorganismos terrestres que formam associações simbióticas multifuncionais com raízes de plantas vasculares, nas quais o fungo facilita a absorção de água e nutrientes pela planta, melhora a tolerância da planta à seca e a organismos patogénicos, e favorece o desempenho da planta em ambientes ricos em metais pesados (por exemplo, solos serpentinicos). Enquanto o papel que os fungos desempenham na diversidade e funcionamento dos ecossistemas é objecto de muitos estudos, a informação existente acerca da estrutura e tamanho do genoma em fungos era, até recentemente, muito escassa para espécies fúngicas não-modelo, apesar da sua importância no estudo da biodiversidade comparativa.

Estudos recentes envolvendo a sequenciação de genomas fúngicos sugerem grandes variações no tamanho do genoma e têm ainda demonstrado uma associação entre o tamanho do genoma e a variação fenotípica. Por exemplo, uma variação no tamanho do genoma parece relacionar-se com a patogenicidade e com um aumento na capacidade de adaptação de certos fungos. Apesar de não ser regra, fungos patogénicos têm frequentemente tamanhos de genoma superiores, o que parece conferir uma maior adaptabilidade e, a longo prazo, uma maior virulência. Um tamanho de genoma maior pode ainda explicar a subsistência de fungos sem uma fase sexual conhecida, e a variação do genoma em si mesma pode promover uma rápida adaptação.

Neste contexto, os principais objectivos desta tese de Mestrado foram estabelecer o tamanho de genoma do fungo ectomicorrízico *Cenococcum geophilum* proveniente de solos serpentínicos e não serpentínicos e investigar a possível relação entre diferentes níveis de ploidia e a sensibilidade ao níquel. Quarenta isolados provenientes de quatro populações do nordeste de Portugal (Bragança) - duas populações serpentínicas e duas não serpentínicas, foram analisados por citometria de fluxo e o tamanho de genoma foi calculado. As estimativas de tamanho de genoma variaram entre 104 e 414 Mbp e foram definidos três níveis de ploidia (haplóide, diplóide e aneuplóide). Em experiências dose-resposta três isolados representantes de cada tipo de solo e de cada nível de ploidia foram expostos a diferentes concentrações de níquel e o incremento de biomassa foi medido ao fim de 21 dias. Perante a exposição ao ní-

quel, não só os isolados serpentínicos e não serpentínicos, mas também os isolados haplóides e diplóides responderam de modo distinto. Estes resultados sugerem que a diploidização poderá contribuir para a adaptação de *Cenococcum geophilum* a solos serpentinicos através de uma maior tolerância ao níquel.

Palavras-chave: *Cenococcum geophilum,* solos serpentínicos, citometria de fluxo, tamanho de genoma, sensibilidade ao níquel, evolução adaptativa.

*Este texto não foi escrito ao abrigo do novo Acordo Ortográfico.

Abstract

Mycorrhizal fungi are a diverse group of soil microorganisms that form multi-functional symbiotic associations with the roots of vascular plants, in which the fungi aid nutrient and water uptake by the plant, improve plant tolerance to drought and pathogens and aid plant performance in environments high in heavy metal content (e.g. serpentine soils). While the important role that fungi play in ecosystem diversity and functioning is under active research, until recently there has been relatively scarce information on genome structure and size for non-model fungal species despite its relevance for comparative biodiversity research.

Recent genome sequencing studies support observations of large variation in fungal genome size, and have further demonstrated an association between genome size and phenotypic variation. For example, genome size variation has been shown to associate with pathogenicity and increased adaptability. Although not the rule, pathogenic fungi often have larger overall genome sizes, which has been suggested to confer adaptability, and over the longer term, greater virulence. A larger genome size may also explain the long-term persistence of fungi having no known sexual stage and genome size variation itself can promote rapid adaptation.

Considering this, the main objectives of this Master thesis were to establish the genome size of the ectomycorrhizal fungus *Cenococcum geophilum* isolated from serpentine and non-serpentine soils and relate its ploidy levels with nickel sensitivity. Forty isolates from four populations in northeastern Portugal (Bragança) were analyzed through flow cytometry and their genome size was assessed. Genome size estimates ranged from 104 and 414 Mbp and three levels of ploidy were established (haploid, diploid and aneuploid). In dose response experiments, three isolates representative of each soil type and ploidy levels were exposed to different Ni concentrations and biomass increment measured after 21 days of growth. A dissimilar response has resulted from exposure of serpentine and non-serpentine isolates, but also of haploid and diploid isolates, to nickel. This result suggests that diploidization can contribute to adaptation of *C. geophilum* to serpentine soils *via* Ni-enhanced tolerance.

Keywords: *Cenococcum geophilum*, serpentine, flow cytometry, genome size, nickel sensibility, adaptive evolution.

GENERAL INTRODUCTION

Serpentine soils

Soil condition is fundamental to the healthiness of terrestrial organisms, such as plants, animals or fungi (Freckman & Virginia 1997, Kruckeberg 2002, Schadt *et al.* 2003). Due to their properties, serpentine soils are ecologically important environments with an extremely high proportion of endemic species (Fig. 1). Worldwide distributed, these biodiversity hotspots are formed by the weathering of ultramafic rocks and typically contain relatively high concentrations of heavy metals such as nickel, chromium and cobalt, have a low calcium-to-magnesium ratio, with calcium levels significantly lower compared to surrounding non-serpentine soils. In addition, they often show low concentrations of essential nutrients such as nitrogen, potassium and phosphorus, and are prone to drought (Brooks 1987, Proctor 1999, Brady *et al.* 2005, Kazakou *et al*. 2008). Altogether, this multifactorial phenomenon responsible for a restriction in plant and microbial growth and selection for metal tolerance is referred as the "serpentine syndrome" (Jenny 1980, Kruckeberg 1984, Amir and Pineau 1998, Brady *et al.* 2005).

Figure 1 General view of a serpentine outcrop in Trás-os-Montes, Portugal (photo by S. C. Gonçalves).

These edaphic conditions present a major challenge to exposed biota and often give rise to a unique flora and possibly mycoflora (Proctor 1999, Panaccione *et al.* 2001), rendering serpentine soils into appealing systems for addressing mechanistic questions of adaptive evolution in natural environments (Brady *et al*. 2005).

Ectomycorrhizal symbiosis

Ectomycorrhizal (ECM) fungi live in symbiosis with trees, mediating soil-plant interactions (Smith and Read 1997). The fungus involves short lateral roots with a mycelial mantle, and penetrates between the plant cells to create a structure called Hartig net (Peterson and Massicotte 2004). The contact with soil is mediated by hyphae extended from the mantle into the soil (Fig. 2). This allows for an increase in the absorption area of the plant with a carbohydrates tradeoff (Smith & Read 2008).

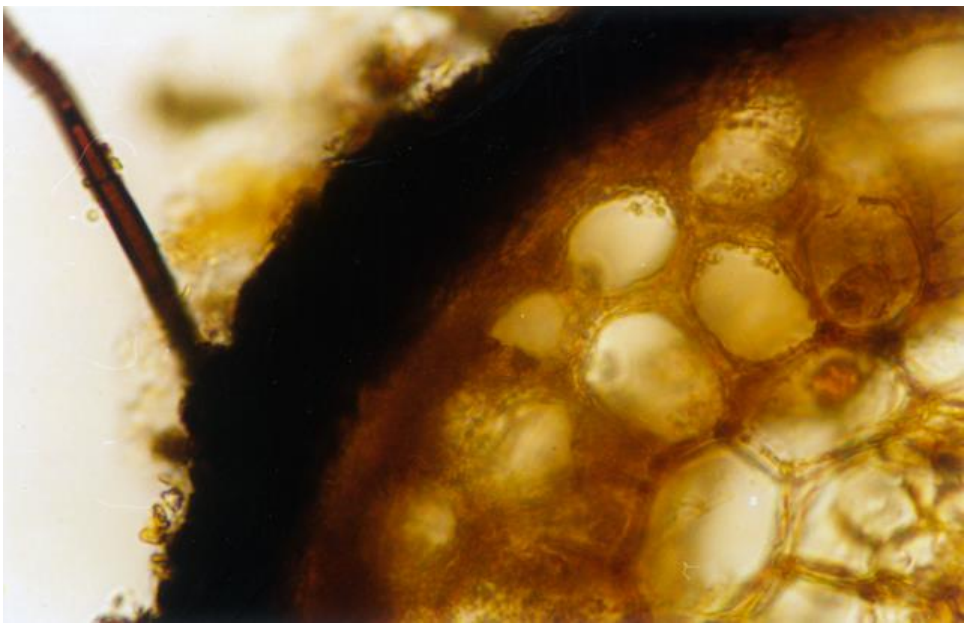

Figure 2 Cross-section of a *Cenococcum geophilum-Quercus ilex* subsp. *Ballota* ectomycorrhiza showing the Hartig net and an emanating hypha

Ectomycorrhizal fungi are also known to assist trees dealing with elevated metal soil concentrations, increasing the tolerance of plant hosts to heavy metals in polluted areas (Wilkins 1991, Godbold *et al.* 1998, Jentschke and Goldbold 2000). Although cellular binding of metals to the hyphal mantle has not been proved experimentally, it has been strongly suggested that extracellular binding to the extraradical mycelium might be significant (Colpaert & Van Assche 1992, 1993). On the other hand, the ameliorating effect can simply be a consequence of the general enhancement of plant fitness. In fact, highly mycorrhizal-dependent trees such as oaks or pines are unlikely to colonize a site that is too toxic to support ECM fungi simply because they depend strongly on ECM fungi for mineral nutrition.

As expected, mycorrhizas associations are present in a huge number of plants growing in serpentine soils (Alexander *et al.* 2007). Even though ECM fungi have the potential to successfully colonize tree roots in serpentine areas, specifically adapted fungi restricted to serpentine habitats have not been reported. Moser and collaborators (2005) expected a lower diversity of ECM fungi in serpentine soils, but no significant differences in mycorrhizal species richness have been found between serpentine and non-serpentine soils.

It has been proposed that some fungal species suffered an environmental pressure in order to adapt and, moreover, dominate metalliferous soils. Some examples include *Phialophora* sp.*, Phialocephala* sp.*, Leptodontidium* sp. (Likar and Regvar 2009), *Cadophora finlandica* (Utmazian *et al.* 2007; Gorfer *et al.* 2009), *Himenoscyphus ericae* (Vralstad *et al.* 2002), *Hebeloma* sp., *Pisolithus* sp. (Turnau *et al.* 1988, Jourand *et al.* 2010), *Rhizopogon* sp. (Turnau *et al.* 1996), *Scleroderma* sp. (Jones and Hutchinson 1986) and *Amanita muscaria* (Gast *et al.* 1988). Despite all these species can be found in contaminated and non-contaminated soils, it has been proposed by several authors that in situations of extreme conditions of metal pollution local ECM fungal populations suffer a selection pressure leading to adaptive evolution of metal tolerance.

Adaptive tolerance to metals has long been proved for plants, but only recently this phenomenon was studied in the Fungi kingdom, with a few fungi showing evidence of adaptive metal tolerance. A small number of species showed an emergence of metal-tolerant ecotypes (adapted to high levels of Al,

Ni, Zn, Cd or Cu): *Pisolithus tinctorius* and *P. albus* (Egerton-Warburton and Griffin 1995, Jourand et al. 2010), *Suillus* species (Colpaert *et al.* 2004, Muller *et al.* 2007, Krznaric *et al.* 2009), *Mortierella polycephala* (Pal *et al.* 2005) and *Cenococcum geophilum* (Gonçalves *et al.* 2009). In the latter example, authors found evidence that *C. geophilum* from serpentine soils have evolved tolerance to Ni in response to the high levels Ni found in serpentine soils.

Cenococcum geophilum

Cenococcum geophilum Fr. is the most common ECM fungus in the world (Trappe 1964, LoBuglio *et al.* 1991, Massicotte *et al.* 1992, Shinohara *et al.* 1999). Described for the first time in 1800 by J. Sowerby as *Lycoperdon graniforme*, the genus name *Cenococcum* was only introduced in 1825 by Elias Fries, which considered *L. graniforme* to be a synonym of *C. geophilum*.

This fungus is distributed worldwide and can be found from frosty areas (Gardes & Dahlberg 1996) to tropical forests (Lee *et al.* 1997), in soils with a pH range from 3.4 to 7.5 (Trappe 1977). It associates with a vast variety of host species, including 200 gymnosperms and angiosperms from 40 genera (Trappe 1964). Ecologically, *C. geophilum* has been proven to be more resilient to drought stress than other ECM fungi (Coleman *et al.* 1989), and was shown to protect plants species from drought when it is involved in ectomycorrhizal symbiosis (Pigott 1982a, b; Wu *et al.* 1999).

The identification of *C. geophilum* relies primarily on mycelium morphology and mycorrhizal characteristics (Trappe 1962, Chilvers 1968, Miller *et al.* 1983). However, information about its taxonomy and genetics is scarce due to the slow growth rate in culture, unavailability of mating tests and inexistence of spores, that constitute important characters in the classification of fungi.

In addition to this remarkable set of ecological features, a significant amount of genetic and genotypic diversity has been detected in *C. geophilum* (Jany *et al.* 2002, LoBuglio and Taylor 2002, Douhan and Rizzo 2005, Wu *et al.* 2005).

Genetic structure of *C. geophilum* populations may reflect ecological and/or physiological diversity, for instance, adaptation to serpentine soils, and namely Ni tolerance (Panaccione *et al.* 2001), or drought tolerance (Jany *et al.* 2002)

Apparently, *Cenococcum* does not produce sexual or asexual spores and although its sexual stage has not been observed yet, during times of environmental stress, it produces abundant sclerotia, which are hypogeous resistant vegetative propagules (Dahlberg *et al.* 1997). Sclerotia can be dispersed by wind, water and animals, including insects and birds (Trappe 1969, Massicotte *et al.* 1992). This fact might explain the worldwide dissemination of the species in spite the absence of spores.

Flow cytometry: introduction

Any physical and/or chemical characteristic of a cell (shape, cytoplasmic granularity, nuclear DNA content) can be measured by flow cytometry (FCM). This technic consists in presenting the particles, one by one within a liquid suspension, to a light source. A few microseconds of exposure allows to collect several parameters (light scatter, fluorescence emissions) at rates of up to 100,000 single particles per second (Shapiro 2003)

Created by Gucker at the behest of US Army, the first working flow cytometer was built in 1947 to identify biological agents in air (Shapiro, 2003), being applied to the detection and counting of red blood cells in saline solution in 1953, by Crosland-Taylor. Nowadays, many research laboratories are equipped with more sensitive flow cytometers which together with the development of better fluorochromes, resulted in high speed analyses of smaller volumes, and allowed its application to many clinical studies (immunophenotyping, leukemia, lymphomas - Brown and Wittwer 2000, Tait *et al*. 2009), plant and animal studies (genome size, detection of specific probes, physiological status – D'hondt *et al.* 2011) and fungal diagnosis (filament formation, metabolic activity, mycosis detection – Hopfer *et al.* 2001, Bradner *et al.* 2003, Meyerson 2008).

Two main advantages of FCM must be considered. Firstly, in a very short time a large number of elements are analyzed, allowing statistically robust results and representative of the population. Plus, it is possible to collect and evaluate approximately 20 parameters of each particle per second. Secondly, in more complex instruments, name flow sorters, once a single particle is analyzed, it can be separated according to its characteristics. This particle can be later studied, since its viability is practically assured (Greilhuber *et al.* 2007).

Light scatter and fluorescence emissions are the parameters detected by the most common flow cytometers. Scatter parameters depend on the size, shape and complexity of the cell. They can be divided in forward (FSC) and side scatter (SSC), according to the angle of the scattered light, either narrow or right, respectively. FSC is dependent on the size and refraction index of the cell whereas SSC depends on the external granularity, internal complexity and shape of the cell. Fluorescence depends on the excitation source and the complexity of the instrument and it can have several fluorescent wavelengths (FL1, FL2, …). Using this parameter, any cellular component to which a fluorescent probe with a wavelength detected by the flow cytometer has been bound, can be detected and the number of particles with that particular component can be quantified (Shapiro 2003).

Flow cytometry applied to fungi

The first attempt to use FCM in fungi is reported in Slater *et al.* (1977). The authors analyzed different stages of the vegetative growth of *Saccharomyces cerevisiae* using flow microfluorometry. The same technique was used by Paau *et al.* (1977a) to compare nucleic acid content in populations of *Rhizobium meliloti* and by Paau *et al.* (1977b) to study different stages of *Escherichia coli*, *R. melioti* and *R. japonicum* growth cycle. One year later, Hutter and Eipel determined the purity of yeast cultures through FCM and Hutter (1978) used immunological fluorescent staining techniques to distinguish yeast strains. In 1994, FCM was used by Smeraldi to analyze the dynamics of *Hansenula polymorpha* peroxisome proliferation and degradation in response to environmental and

genetic factors. Recently, a new multiplexed method based on FCM was developed by Rai (2012) to detect harmful mycotoxins that frequently contaminate the grain. Over the last decades, FCM was also used to estimate genome size and ploidy level in some fungal organisms (Dvorak *et al.* 1987, Carr and Shearer 1998, Almeida *et al.* 2007, Anderson *et al.* 2010).

Nuclear DNA content

For a long time researchers have been seeking to understand the biological basis behind the large variation in genome size observed in many organisms and search for ecological, evolutionary, and taxonomic interpretations. The observation of the non-correlation between the amount of nuclear DNA and the organismal complexity is referred as the "C-value enigma". The C-value concept was firstly assigned by Swift in 1950 and was relative to “constant” DNA content, i.e., the total DNA content that was characteristic of a particular genotype (Swift 1950). Nowadays, C-value is understood as half the DNA content of a somatic cell (2C-value). Several hypotheses were raised to try to explain the enormous variation in genome size, being the nucleotype theory in the spotlight. Nucleotype theory was originally proposed by M. D. Bennett, who defended that the DNA of an organism could influence its phenotype not only through its informational content (genotype), but also through the physical effects of its mass and volume (nucleotype) (Bennett 1971).

Genome size can be expressed in two different ways: mass units (picograms, pg), or base pairs numbers (bp). Both units are interconvertible through the following formula, recently corrected by Doležel *et al* (2003):

$$\text{DNA content (bp)} = (0.978 \times 109) \times \text{DNA content (pg)}$$
$$\text{DNA content (pg)} = \text{DNA content (bp)} / (0.978 \times 109)$$

In the particular case of fungi, despite there are some studies focused in evaluating the genome size variation in particular groups, it was only recently that FCM was used for this purpose. The recent interest in such studies was fueled by the interest to sequence fungal genomes as sequencing efforts al-

ready pointed for a large variation in the sizes of fungal genomes (Cuomo and Birren 2010, Martin *et al.* 2010, Spanu *et al.* 2010, Goodwin *et al.* 2011). Also, it has been proposed that genome size variation in fungi result of an adaptation to their lifestyle (Martin *et al.* 2010), with smaller genomes corresponding to free-living and pathogenic species (Dujon 1996), whereas larger and greatly expanded genomes refer to obligatory parasitic/mutualistic species (Martin *et al.* 2010, Spanu *et al.* 2010).

In order to compile the available data concerning genome size in fungi, in the 20th March 2005 the Fungal Genome Size Database (http://www.zbi.ee/fungal-genomesize/) was launched. In its current version it includes 1,298 records covering 739 species from 40 orders and 335 genera. The present knowledge of genome size in fungi is represented in Figure X. In comparison with the animal and plant genome sizes, fungi display very small genomes:~90% of the estimations range from 10 to 60 Mb (Fig. 3).

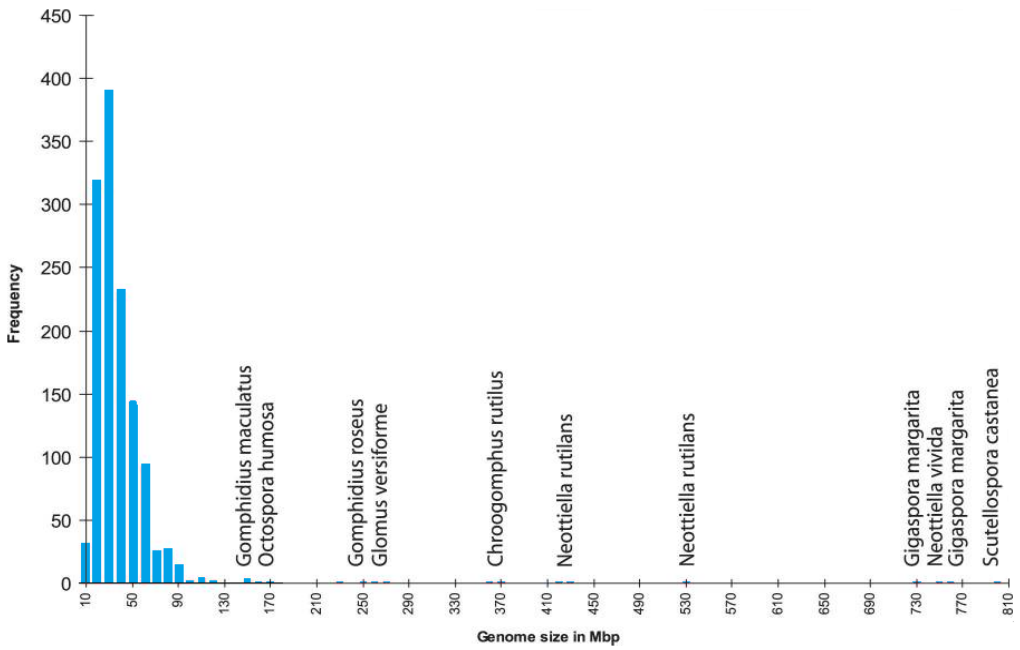

Figure 3 Cross-section of a *Cenococcum geophilum* ectomycorrhiza extending its hyphae to *Quercus ilex* subsp. *Ballota*

Study system, thesis objectives and layout

C. geophilum has become the focus of increased research effort, partly due to its broad geographic and ecological distribution (LoBuglio 1999). Although multilocus genotype assessments suggest the occurrence of recombination and a possible cryptic sexual cycle (Taylor *et al*. 1999, Lobuglio and Taylor 2002, Douhan *et al*. 2007), a sexual stage has so far been assumed to be absent in *C. geophilum* (Fernandez-Toiran and Agueda 2007, Spatafora *et al*. 2012). While this absence would imply clonal reproduction and a relatively low genetic diversity, various studies have demonstrated an unexpectedly high level of genetic diversity within and between *C. geophilum* populations (Pannacione *et al*. 2001, Wu *et al*. 2005, Douhan *et al*. 2007, Gonçalves *et al*. 2007). *Cenococcum geophilum* has been shown to be locally adapted to serpentine soil conditions, namely to high Ni concentrations (Gonçalves *et al*. 2007, 2009). Although many studies have been performed on serpentine flora and systematics, natural history, ecology and physiology, possible relationships between adaptation to serpentine soil conditions and genome size variation have not received much attention. Nevertheless, independent polyploid evolution in serpentine populations of *Knautia arvensis* (Dipsacaceae) that promoted further evolution of serpentine lineages has recently been reported (Kolař *et al*. 2012).

This Master thesis was developed within the framework of the research project CENECOGEN (FCT funded; PTDC/BIA-BEC/100733/2008) which aims at dissecting the genetic basis of adaptive serpentine tolerance of the ectomycorrhizal fungus *Cenococcum geophilum*. Because the project involves the determination of the fungus genome sequence, the first objective of this thesis was to establish the genome size of the species *C. geophilum*, a prior step to genome sequencing. The second objective was to investigate the relationship between genome size and serpentine adaptation.

In accordance with the proposed objectives, this Master thesis was organized in two main chapters. In **Chapter One** ("Large and variable genome size unrelated to serpentine adaptation but supportive of cryptic sexuality in *Cenococcum geophilum*"), *Cenococcum geophilum* genome size was estimated and its variation

assessed between and among populations from serpentine and non-serpentine areas. In **Chapter Two** ("Soil of origin and ploidy level affect *in vitro* Ni tolerance in *Cenococcum geophilum* from serpentine and non-serpentine soils"), Ni dose-response experiments treatments were performed to investigate the Ni tolerance of serpentine and non-serpentine isolates, taking into account a possible effect of ploidy level.

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CHAPTER ONE

Large and variable genome size unrelated to serpentine adaptation but supportive of cryptic sexuality in *Cenococcum geophilum**

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INTRODUCTION

Nuclear genome sizes vary tremendously between eukaryotic species (Whitney and Garland (2010) report a range spanning eight orders of magnitude) and this variation is due to a multitude of processes, including polyploidization, gene duplication and amplification of transposable elements (Soltis and Soltis 1999, Kazazian 2004, Cui *et al*. 2006). Although these mutational processes can induce structural and functional changes that are assumed to promote further evolution and adaptation in plants, animals and fungi, it is currently still under debate whether the variation in genome size between species is predominantly the result of adaptive or of neutral processes (Whitney and Garland 2010).

Besides variation in genome size between species, variation within species is also commonly observed. This within-species genome plasticity has been well studied in several fungal species where it has been found to be associated with life cycle or cellular differentiation and with changing environmental conditions (Albertin and Marullo 2012). E.g. *Candida albicans* is a diploid yeast that frequently enters a cryptic mating or parasexual cycle in which somatic cells fuse and the resulting tetraploid cells undergo a random loss of chromosomes. As a result, a variety of ploidy levels, including aneuploidy, can be observed among *C. albicans* isolates (Ibrahim *et al*. 2005). *Candida glabrata* on the other hand is a haploid fungal species that displays frequent changes in its chromosome complement in relation with pathogenicity and a changing environment (Polakova *et al*. 2009). Specific environmental factors that have been shown to affect fungal genome size include salt stress (Dhar *et al*. 2011), fungicide treatments (Welker and Williams 1980), heat shock treatments (Hilton *et al*. 1985) and host-pathogen interactions (Raffaele and Kamoun 2012).

We aimed to investigate genome size in the ectomycorrhizal ascomycete *Cenococcum geophilum* Fr. and assess whether it is affected by serpentine edaphic conditions. *C. geophilum* has become the focus of increased research effort, partly due to its broad geographic and ecological distribution (LoBuglio 1999). Although multilocus genotype assessments suggest the occurrence of recombination and a possible cryptic sexual cycle (Taylor *et al*. 1999, Lobuglio

and Taylor 2002, Douhan *et al*. 2007), a sexual stage has so far been assumed to be absent in *C. geophilum* (Fernandez-Toiran and Agueda 2007, Spatafora *et al*. 2012). While this absence would imply clonal reproduction and a relatively low genetic diversity, various studies have demonstrated an unexpectedly high level of genetic diversity within and between *C. geophilum* populations (Pannacione *et al*. 2001, Wu *et al*. 2005, Douhan *et al*. 2007, Gonçalves *et al*. 2007).

C. geophilum has been shown to be locally adapted to serpentine soil conditions (Goncalves *et al*. 2007, 2009). Although many studies have been performed on serpentine flora and systematics, natural history, ecology and physiology, possible relationships between adaptation to serpentine soil conditions and genome size variation have not received much attention. Nevertheless, independent polyploid evolution in serpentine populations of *Knautia arvensis* (Dipsacaceae) that promoted further evolution of serpentine lineages has recently been reported (Kolař *et al*. 2012).

Here, we report on the genome size of *C. geophilum* and its naturally occurring variation as determined by flow cytometry analysis of 40 isolates obtained from two serpentine and two nonserpentine populations in the northeast of Portugal. We first present data describing variation in genome size occurring in this fungal species and discuss the implications of our findings in the context of adaptive evolution of serpentine tolerance. In addition, we show that our data provides support for a possible cryptic mating or parasexual cycle in *C. geophilum*.

MATERIALS AND METHODS

Field sites, fungal isolation and culturing

Two forest areas from northeastern Portugal (Trás-os Montes) were selected to collect *Cenococcum geophilum* sclerotia, a resistance structure composed of a compact mass of mycelium. Samples were taken from two serpentine (Serra da Nogueira - 41°47'58.975 N, 6°54'15.545 W; Espinhosela - N 41°51'22.126 W 06°50'424) and two non-serpentine (Rabal - N 41°52'14.875, W 06°44'40.000; Aveleda - 41°52'8.41 N, 6°41'58.97 W) areas, and collected at least five meters apart, to ensure sampling of different individual genets.

Soil samples went through a sieving and water-washing process, allowing hand-picking and subsequent sterilization of the sclerotia in 30% hydrogen peroxide, for 10-20 seconds (Douhan & Rizzo 2005). Sclerotia were grown and germinated in Potato Dextrose Agar (Difco, Sparks, MD, USA) with streptomycin (0.2 mg l^{-1}) and then transferred potato dextrose agar (PDA; Difco, USA) with no antibiotic. Growing stage occurred for at least on month at room temperature in the dark.

In total, we used forty isolates of *Cenococcum geophilum*, twenty from serpentine areas and twenty from non-serpentine areas. Table 1 summarizes the origins of the studied isolates. All the isolates were kept in our collection in PDA medium.

Table 1 Origin site of the isolates of *Cenococcum geophilum* studied.

Population	Nogueira	Aveleda	Espinhosela	Rabal
Isolate ID	A4.1	B5.1	C6.4	D3.3
	A8.1	B22.1	C46.2	D22.1
	A14.1	B26.1	C60.1	D32.1
	A73.1	B35.1	C68.1	D33.1
	A75.1	B44.1	C73.1	D35.2
	A76.1	B48.1	C79.2	D38.1
	A90.3	B49.1	C80.1	D44.1
	A91.3	B51.1	C82.2	D47.1
	A97.1	B57.3	C86.8	D67.1
	A99.1	B59.1	C92.1	D70.1

Genome size estimation

The nuclear DNA content of *Cenococcum geophilum* was estimated by flow cytometry using fresh mycelium from young colonies. Propidium iodide-stained nuclei were extracted from ten *in vitro* cultured isolates per population and the reference isolate Cg 1.58. For each isolate, three independent replicate measurements were performed. Mycelium was collected with a needle and added to a Petri dish with 1 mL of modified WPB buffer (0.2 M Tris-HCl, 4 mM $MgCl_2.6H_2O$, 2% Triton X-100, 2 mM disodium EDTA, 86 mM NaCl, 20 mM metasulfite, 4% PVP-10, [pH 7.5]; Loureiro *et al.* 2007). Nuclei were released following the chopping procedure of Galbraith *et al.* (1983) by chopping approximately 10 mg of mycelium with a razor blade together with 50 mg of fresh leaf tissue of *Raphanus sativus* (internal reference standard with 2C = 1.16 pg or 1,135 Mb; Dolezel *et al.* 1998) to ensure that nuclei of both species were exposed to identical chemical and mechanical conditions.

The nuclear suspension was filtered through a 30 µm nylon filter to remove large fragments and 50 µg/mL of propidium iodide (PI, Fluka, Buchs, Switzerland) and 50 µg/mL of RNase (Fluka, Buchs, Switzerland) subsequently added to stain the nuclei and to prevent staining of double-stranded RNA, respectively. After incubation for 5 minutes, the fluorescence intensity of at least 2000 nuclei per sample was analyzed using a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany), equipped with a green solid state laser for PI excitation. The G_0/G_1 peak of the standard was set to channel 720, with the amplification system kept at a constant voltage and gain throughout the experiment.

ITS Sequencing

To confirm species identification isolates representative of the main ploidy groups were sequenced for the ITS region and compared to known sequences within Genbank. All remaining isolates were confirmed by visual identification. DNA extraction followed the procedure outlined in the Dneasy Plant Minikit (Qiagen) with modifications: Mycelium was cultured on PDA, scraped into a 2 ml microcentrifuge tube and subsequently ground with a sterlised micropestle in

liquid nitrogen and sand. Samples were lysed in 400 µl lysis buffer AB1 and 4 µl RNase at 75^{o} C, for three hours, with occasional vortexing. Amplification was carried out using the primer pair ITS1F/ITS4 (White et al. 1990, Gardes & Bruns 1993) in a 50 µl reaction volume containing: approximately 20 ng of template DNA, 2 mM MgCl2, 0.25 mM each dNTP, 0.5 µM each primer, and 2.5 U of Taq DNA polymerase (Fermentas) with the recommended buffer (1x KCl Buffer). PCR conditions followed: initial denaturation at 96 ºC for 2 mins; amplification over 20 cycles of 94^{o} C for 30s, 56^{o} C for 1 min, and 72^{o} C for 1 min; extension at 72^{o} C for 10 mins, and held at 4^{o} C. The PCR products were commercially sequenced in both directions (StabVida, Portugal), with ITS1F and ITS4 as sequencing primers. Sequences were edited and aligned using Bioedit ver 7.1.3.0 (Hall *et al.* 1999).

Data analysis

Histograms of propidium iodide fluorescence intensity were obtained and evaluated using FloMax software v2.4d (Partec GmbH, Münster, Germany), and the genome size of each sample was determined according to the following formula (in pg):

$$DNA\ content\ of\ sample = \frac{mean\ G_0/G_1\ fluorescence\ of\ sample\ nuclei}{mean\ G_0/G_1\ fluorescence\ of\ reference\ nuclei}\ x1.16$$

The reliability of the genome size measurements were certified by evaluating the quality of the flow cytometry histograms based on the coefficient of variation (CV) of the G_0/G_1 peaks and the background debris, and the CV of the genome size estimation of each isolate based on three independent measurements.

As ANOVA assumptions were not met, differences in genome size between populations were evaluated by a Kruskal-Wallis one-way analysis of variance by ranks (*H*-test) with a significance level of $P < 0.05$ using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA). A contingency test was performed to assess the association between ploidy level and ecological background using the Fisher's Exact Test for count data in the statistical program R (ver. 2.15.1, R Core Team 2012). Genome size data was downloaded from the Fungal Genome Size Database (FGSD, Kullman *et al.* 2005) and included all records available to October 2012, with a mean value calculated for each species upon the occurrence of multiple entries.

RESULTS

Clear symmetrical G_0/G_1 fluorescence intensity peaks of both the fungal and the plant reference nuclei were obtained (Fig. 4), with a mean CV value of the fungal G_0/G_1 peaks of 7.34 %, falling within the range of values reported in similar works (Sędzielewska *et al.* 2011). No mean CV measures of individual genome size estimations exceeded 10 % (Appendix A)**.** The endopolyploid nature of *R. sativus* (Kudo and Kimura 2001) did not affect the analysis as only the peak corresponding to the 2C nuclei, which is used for genome size estimation, appeared on the scale set (Fig. 4).

Table 2 Summary of genome sizes determined by flow cytometry analysis and presumed ploidy levels of *Cenococcum geophilum* isolates collected from two serpentine (S, Espinhosela and Nogueira) and two non-serpentine (NS, Aveleda and Rabal) sites in Portugal; SD, standard deviation, *n*, number of individuals. *One isolate was mixoploid (diploid and haploid nuclei present).

Population	Soil type	Ploidy level	Nuclear DNA content		*n*
			Mean ± SD (pg)	Mean ± SD (Mbp)	
Espinhosela	S	haploid	0.199 ± 0.01	194 ± 10	9
		diploid	-	-	-
		aneuploid	0.283 ± 0.03	276 ± 29	1
Nogueira	S	haploid	0.218 ± 0.01	213 ± 10	9
		diploid	0.410 ± 0.03	401 ± 29	1
		aneuploid	-	-	-
Aveleda	NS	haploid	0.213 ± 0.01	209 ± 10	8
		diploid	0.421 ± 0.03	411 ± 29	1
		aneuploid	0.361 ± 0.01	353 ± 10	1
Rabal	NS	haploid	0.201 ± 0.01	196 ± 10	8
		diploid*	0.417 ± 0.04	409 ± 39	2
		aneuploid	-	-	-
All populations		haploid	0.208 ± 0.01	203 ± 10	34
		diploid	0.416 ± 0.01	414 ± 10	4
		aneuploid	0.322 ± 0.06	315 ± 59	2

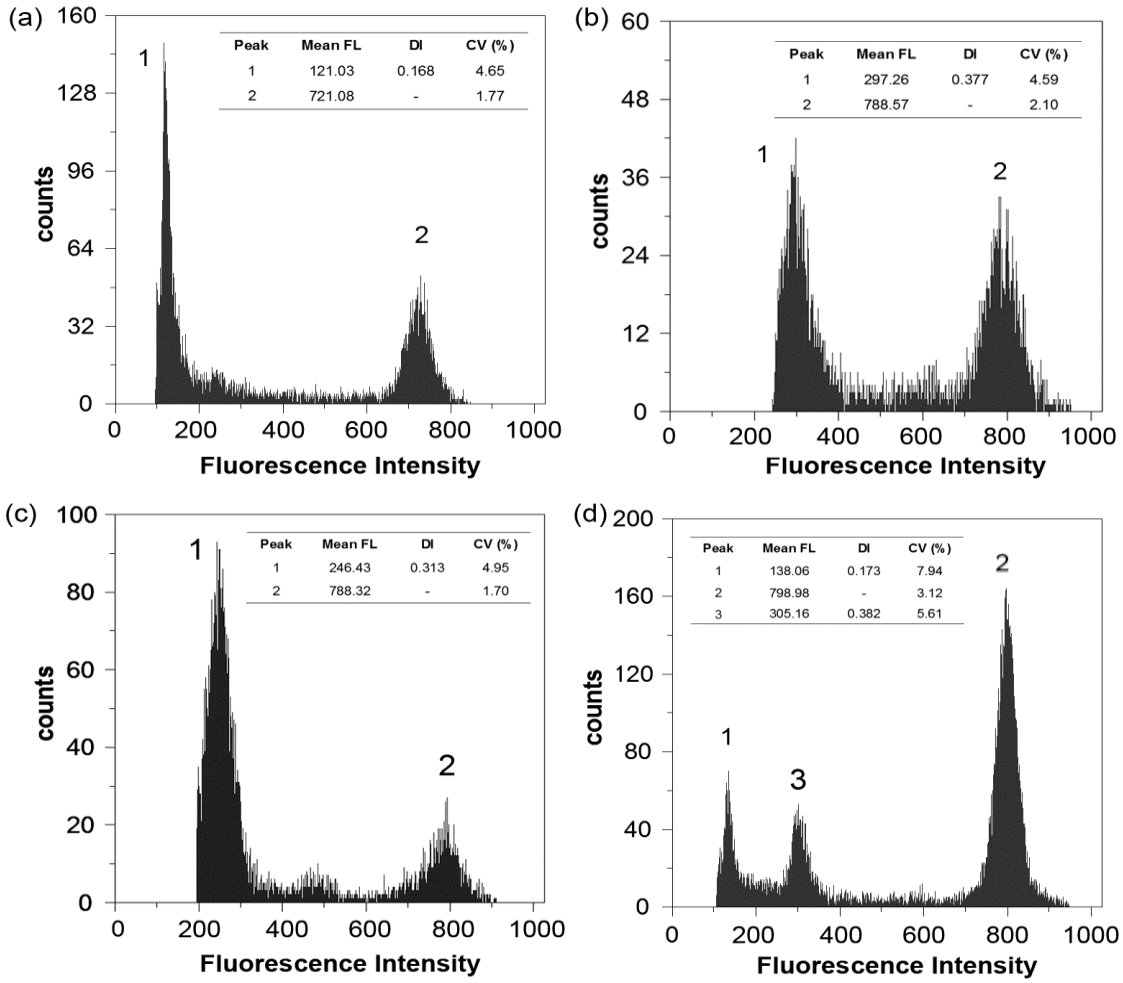

Figure 4 Histograms of relative fluorescence intensities obtained through simultaneous flow cytometry analysis of propidium iodide stained nuclei of *Cenococcum geophilum* (peaks 1 and 3) and *Raphanus sativus* cv. Saxa (peak 2; internal reference standard with 2C = 1.16 pg or 1,135 Mbp). Histograms are shown for the following ploidy classes: a) haploid (isolate B48.1), b) diploid (isolate B5.1), c) aneuploid (isolate B57.3) and d) mixoploid (isolate D70.1). Peaks are marked as: 1, *C. geophilum* nuclei at the G_0/G_1 phase; 2, *R. sativus* nuclei at the G_0/G_1 phase; 3, diploid *C. geophilum* nuclei at the G_0/G_1 phase and haploid nuclei at the G_2 phase. The inset table provides information on the mean channel number (the mean fluorescence intensity of stained nuclei), the DNA index (DI = mean channel number of sample/mean channel number of reference standard) and the coefficient of variation (CV, %) of each peak.

The mean DNA content per nucleus across the triplicate measurements for each isolate varied between 0.170 ± 0.010 pg (166 ± 10 Mb) and 0.439 ± 0.040 pg (430 ± 39 Mb), with an overall median and median absolute deviation (MAD) across isolates of 0.209 ± 0.012 pg (205 ± 12 Mb) (Table 2, Appendix A and Fig. 5). As the distribution of the average DNA content per isolate is skewed towards the lower end of its range and *C. geophilum* is assumed to be a haploid organism with n = 6 chromosomes (Portugal et al. 2002), the majority of the isolates (85 %; Appendix A, Fig. 5) included in this study are presumed to be haploid with an average mean DNA content per nucleus of 0.208 ± 0.016 pg (203 ± 16 Mb).

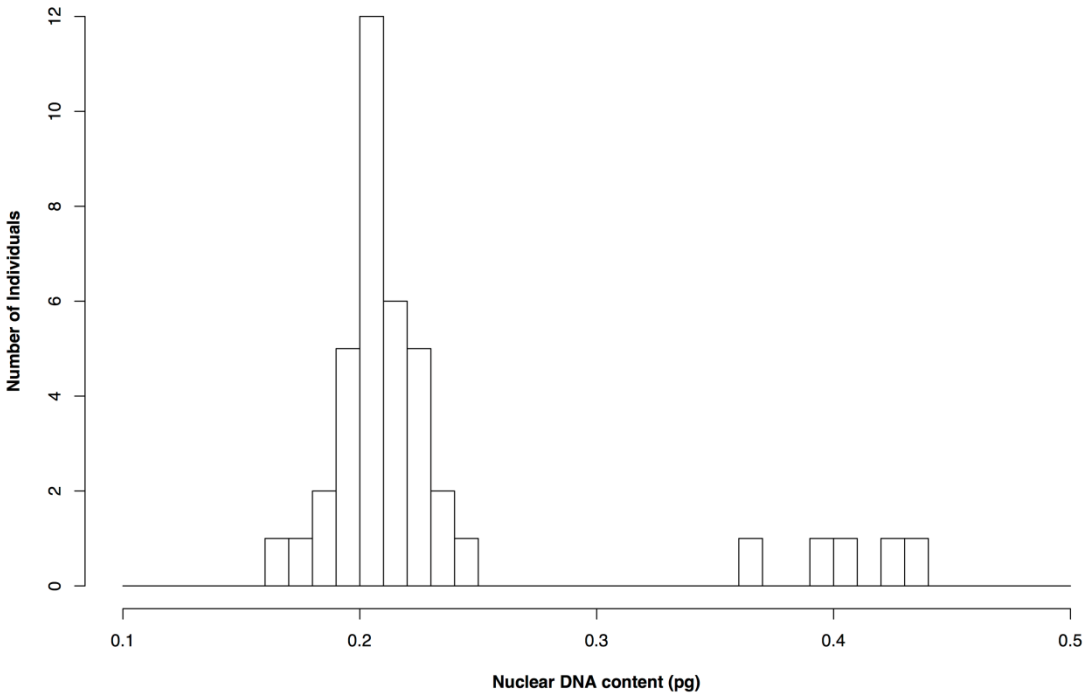

Figure 5 Genome size variation in *C. geophilum*. Histogram of nuclear DNA content in pictogram (pg) estimates for 40 isolates of *Cenococcum geophilum* estimated by propidium iodide cytometry.

An estimated DNA content of 0.246 ± 0.027 pg (241 ± 27 Mb) was obtained for the reference isolate Cg 1.58 (Appendix A), in accordance with initial estimations based on preliminary genome sequencing results (M. Peters, personal communication). Six isolates had genome sizes suggesting variation in ploidy

level within *C. geophilum*, with three isolates giving diploid estimates and two isolates giving aneuploid estimates (Fig. 4, Appendix A). Interestingly, one isolate (D70.1) was found to be mixoploid and displayed an approximately equal amount of haploid and diploid nuclei (Fig. 5d, Appendix A)**.**

DNA sequences of ITS in isolates of each of the three ploidy classes (haploid: isolate C79.2, diploid: isolate A99.1, and aneuploid: isolate B57.3; Appendix A) were obtained to rule out misidentification of our fungal cultures and therefore taxonomic diversity having caused the observed variation in genome size. Average pairwise sequence similarity between the three ITS1 sequences was 97 %, indicating a close phylogenetic relatedness of isolates with different ploidy levels, and sequence comparisons with all available fungal DNA sequences in the NCBI and UNITE databases resulted in sequences from other cultured *C. geophilum* isolates to be identified as best hits (with 100 % query coverage, 99 % maximum identity and E values≤$2e^{-63}$).

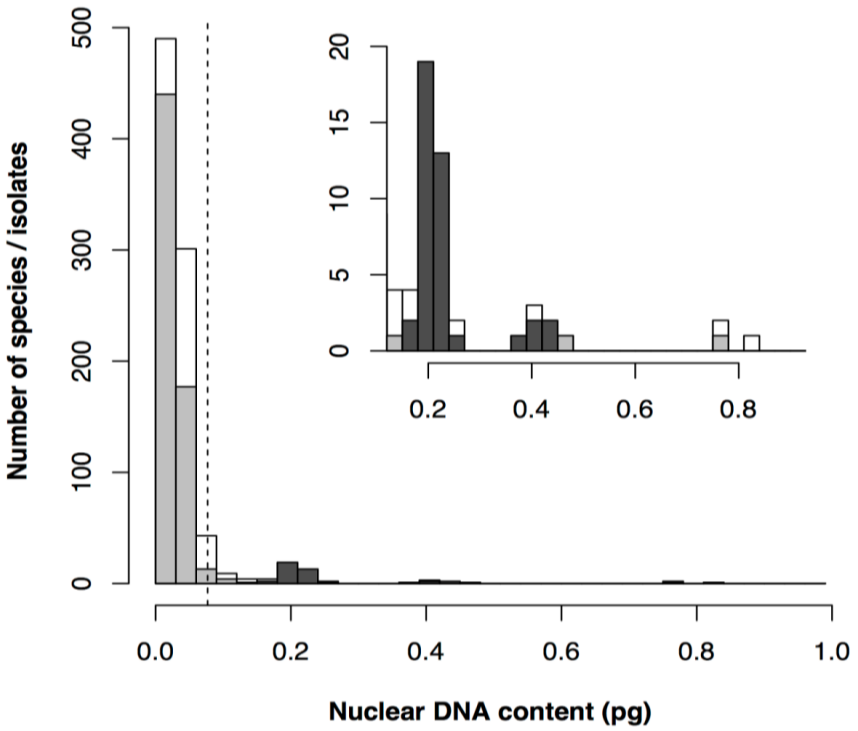

Figure 7 Genome size variation among fungal species. Mean genome size in picogram (pg) of each species reported in the Fungal Genome Size Database (FGSD; open bars) with the distribution of Ascomycota highlighted (light grey) and the genome size estimates of 40 *C. geophilum* isolates (dark grey). Inset graph: detailed view of the size distribution for the upper range, including *C. geophilum*. The 95th percentile of all species reported in the FGSD is marked as a vertical dashed line (0.0795 pg).

Comparison of our estimates, of both presumed haploid and diploid individuals, with genome size estimates submitted to the FGSD reveals *C. geophilum* to have a genome size distribution in excess of the 95th percentile of the genome size distribution of all fungal species (Fig. 7)**.** The mean genome size of presumed haploid *C. geophilum* isolates within each population ranged between 0.199 pg (195 Mb) and 0.218 pg (213 Mb) (Table 2), with no significant difference in genome size between the populations (Kruskal Wallis, $H_{3,31}$ = 4.438, P = 0.218). In addition, no significant association was found between ploidy level and edaphic background (Fishers exact test, P = 0.604).

DISCUSSION

A growing body of evidence indicates large variation in genome size in the fungal kingdom. While about 90 % of estimations range from 10 to 60 Mb (Gregory *et al.* 2007), extreme haploid genome sizes of up to 795 Mb have been reported for *Scutellospora castanea* (Glomeromycota; Hosny *et al.* 1998, Hijri and Sanders 2005). The Ascomycota are well represented throughout this range, with estimates of 7.7 Mb for *Bulgaria inquinans* (order Helotiales; Weber 1992) to 750 Mb for *Neottiella vivida* (order Pezizales; Kullman 2002).

Considering the range of genome sizes observed in the Ascomycota, our estimates of an average haploid genome size of 203 Mb in *C. geophilum* indicate that the genome size of this species is much larger than expected a priori and this holds even when extending our comparison to the whole fungal kingdom (Fig. 7). Although large genome sizes are generally recorded for highly polyploid species (e.g., *N. vivida* has a ploidy level of 70; Kullman 2002), exceptionally large genome sizes have been observed in other haploid species (e.g., the haploid arbuscular mycorrhizal species *Glomus etunicatum* has a genome size of 750 Mb; Hijri and Sanders 2005).

In a recent analysis of the underlying processes associated with genome size expansion within the Ascomycota, Kelkar and Ochman (2012) suggest that genetic drift, reflected through a decrease in gene density and a proliferation of

introns, has played a significant role across many lineages on broad evolutionary time scales. The large genome size of *C. geophilum* might therefore result from ancestral genome size expansion driven by genetic drift, which would lead to a repeat-rich and relatively gene-poor genome. However, selective and neutral processes together are associated with genome size expansion within some lineages as observed by Kelkar and Ochman (2012) and others (Martin *et al.* 2010). Future analyses of the genome sequence of *C. geophilum* may provide more insights into whether its surprisingly large genome size is the consequence of genetic drift alone or in combination with selective processes.

Besides variation in genome size among the haploid *C. geophilum* isolates, variation in ploidy level was observed as four *C. geophilum* isolates were considered to be diploid (including the observed mixoploid) and two to be aneuploidy based on our genome size estimations. Such intraspecific variation in genome size, including the variation among the isolates of a given ploidy level, is common among fungi. This variation often results from chromosome length polymorphisms and/or from gain/loss of complete chromosomes or sets thereof, as has been demonstrated in *Cronartium quercuum* (Anderson *et al.* 2010), *Cryptococcus neformans* (Hu et al. 2011), and *Saccharomyces cerevisiae* (Codón *et al.* 1998; Albertin *et al.* 2009).

Despite the strong selection pressure that is expected in serpentine soils and a report of possible influences of serpentine on the evolution of polyploids in *K. arvensis* (Kolář *et al.* 2012), we did not find significant differentiation in genome size between the *C. geophilum* populations included in this study, suggesting that genetic and demographic processes are more important in shaping the genome size variation of this species than environmental selection in the form of home soil chemistry. While these results suggest that variation in ploidy level is not related to serpentine tolerance in *C. geophilum*, the relatively large genome size - providing the potential for local adaptation to develop in vastly different environments - may still contribute to the wide ecological breadth, including the occurrence in serpentine soils, reported for this species.

The identification of diploid and mixed haploid/diploid *C. geophilum* isolates may indicate the occurrence of a cryptic mating or parasexual cycle in this presumed asexual fungal species. As first described for *Aspergillus nidulans* (Pon-

tecorvo *et al.* 1953), the standard parasexual cycle starts with the formation of a heterokaryon (plasmogamy) followed by a fusion of the two haploid genomes (karyogamy). The resulting diploid nuclei divide mitotically along with remaining haploid nuclei and may form a discrete sector or even propagate as separate diploid strains. However, the diploid nuclei may be unstable and produce haploid or aneuploid segregants through mitotic recombination and chromosomal nondisjunction. Our observation of diploid and mixed haploid/diploid *C. geophilum* isolates thus supports an earlier suggestion of a cryptic mating or parasexual cycle in *C. geophilum* based on the indirect observation of genetic recombination using DNA sequence or genetic marker data (Lobuglio and Taylor 2002, Douhan *et al.* 2007). A sexual stage in *C. geophilum* was also recently proposed by Spatafora *et al.* (2012) based on the large genetic diversity detected in this species and a close phylogenetic relationship with *Glonium*, for which a sexual stage has been identified. Assexual reproduction in fungi can be geographically isolated and therefore difficult to detect (O'Gorman *et al.* 2009; Spatafora *et al.* 2012), it is not surprising that we found only 6 in 40 isolates to deviate from a presumed haploid condition. However, the existence of a parasexual cycle is not the only possible explanation for the observed variance in ploidy level. Alternatively, this variance may be the result of an endopolyploidization process that involves recurrent cycles of DNA replication without cellular division and produces an increase of the genome content in the somatic line or it may reflect the existence of phylogenetically diverged lineages within a *C. geophilum sensu lato* species complex that was proposed by Douhan *et al.* (2007).

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CHAPTER TWO

Soil of origin and ploidy level affect *in vitro* Ni tolerance in *Cenococcum geophilum* from serpentine and non-serpentine soils

INTRODUCTION

Serpentine soils provide a remarkable study system for the comprehension of the population biology and physiology of ectomycorrhizal (ECM) fungi colonizing trees on natural metalliferous sites (Panaccione *et al.* 2001) and, although still limited, a growing number of research studies have been made concerning ECM fungi in these environments (Maas and Stuntz 1969, Moser *et al.* 2005, 2009; Panaccione *et al.* 2001, Bearley 2006, Urban *et al.* 2008, Gonçalves *et al.* 2009, Branco 2010, Branco & Ree 2010). Moser *et al.* (2005) found diverse ECM fungi communities in both serpentine and non-serpentine soils, with *Cenococcum geophilum* being the most abundant species. *Cenococcum geophilum* was found at all replicate sites (serpentine and non-serpentine), which suggested the occurrence of serpentine-tolerant ecotypes in serpentine soils. In another study, *C. geophilum* has also been isolated from *Pinus virginiana*, growing both in serpentine soils and non-serpentine soils in Maryland, USA, and the authors found evidence of genetic divergence between serpentine and non-serpentine isolates consistent with the presence of serpentine-tolerant ecotypes (Panaccione *et al.* 2001). More recently, Gonçalves *et al.* (2007, 2009) examined the *in vitro* tolerance to Ni of *C. geophilum* isolates from serpentine and non-serpentine soils. Serpentine isolates exhibited significantly higher tolerance to Ni than non-serpentine ones suggesting adaptation in response to natural metal contamination of serpentine soils.

In Chapter 1, genome size estimates were obtained for *C. geophilum* coming from serpentine and non-serpentine populations, comprising a range from 194 to 414 Mbp and three ploidy levels. Considerations about the causes for genome size variation have been largely discussed and it has been proposed that this variation could possibly have an adaptive significance in spite of resulting from non-coding DNA (Doolittle & Sapienze 1980). According to the nucleotypic theory, the condition of the nucleus affects organism phenotype, independently of the informational content of the DNA (Bennet 1971). Ecological, taxonomically and evolutionary interpretations have long been searched in order to understand the biological basis of genome size diversity and its adaptive value. In fact, associations between genome size and a wide range of morphological, cytological, developmental, physiological, reproductive, and ecological

traits have been searched and, in some cases, found (Bennett & Leitch 2005, Gregory 2005). Differential selection across ploidy levels has been observed in different abiotic environments (Dhar *et al.* 2011) and increased ploidy has been suggested to provide a selective advantage in adaptation to new environmental conditions (Pawlowska and Taylor 2004, Ma *et al.* 2009). However, despite the strong selection pressure that is expected in serpentine soils and a report of possible influences of serpentine on the evolution of polyploids in *K. arvensis* (Kolář *et al.* 2012), we did not find significant differentiation in genome size between the *C. geophilum* populations included in our study, suggesting that genetic and demographic processes are more important in shaping the genome size variation of this species than environmental selection in the form of home soil chemistry (Chapter 1).

In this chapter, our objective was to evaluate the Ni sensitivity in six *C. geophilum* isolates, three serpentine and three non-serpentine. Isolates were among those screened for genome size and included isolates from the three ploidy levels that were established (Chapter 1). Based on previous results (Chapter 1; Gonçalves *et al.* 2007, 2009), we predicted that isolates from serpentine sites will exhibit lower sensitivity to Ni than isolates from the non-serpentine sites, independently of the ploidy level.

MATERIAL AND METHODS

Fungal material

The *C. geophilum* isolates examined in this study were the ones previously analyzed by flow cytometry (Chapter 1). A total number of six isolates were chosen, and considering each ploidy level (haploid, diploid and aneuploid) and soil of origin (serpentine and non-serpentine) (Table 3). Detailed information about the characteristics of the sites and description of the methods of isolation and maintenance of the fungi can be found in Chapter 1.

Table 3 Isolates of *Cenococcum geophilum* used in this study according with the ploidy level and soil type

Soil type	Ploidy level		
	Aneuploid	n	2n
Serpentine	C6.4	A73.1	A99.1
Non-serpentine	B57.3	B44.1	D33.1

Ni tolerance screening

Metal tolerance was tested in liquid modified Fries medium (Fries 1978) containing (mM): D-glucose, 28; ammonium tartrate, $(NH_4)_2C_4H_4$, 5.4; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.4; $CaCl_2.2H_2O$, 0.2; NaCl, 0.3. Microelements included (µM): $FeCl_2.6H_2O$, 4.0; $ZnSO_4.7H_2O$, 3.0; $MnSO_4.H_2O$, 6.0; $CuSO_4.5H_2O$, 0.8; $Na_2MoO_4.2H_2O$, 0.8; myo-inositol, 56; biotin, 0.1; pyridoxine-HCl, 0.5; riboflavin, 0.3; nicotinamide, 0.8; p-amino-benzoic acid (PABA), 0.7; thiamine-HCl, 0.3 and Ca-pantothenate, 0.2. Media were adjusted to pH 5.5 and autoclaved for 15 min at 121 ºC. Vitamins and myo-inositol were previously sterilized by filtration (0.2 µm) and added to the medium after cooling and immediately before its utilization. Seven Ni treatments were established; the final metal concentrations (0, 0.5, 2, 10, 20, 50, and 100 µg g^{-1} Ni) were obtained by adding aliquots of a stock solution containing 2000 µg g^{-1} Ni (supplied as nickel sulphate (Ni-

$SO_4.7H_2O$) to the growth medium. For each Ni concentration five replicates were made.

Cylindrical plugs with 4 mm diameter were extracted from active growing colonies with a sterilized borer and placed on fresh PDA medium plates to obtain uniform inocula. When visible hyphal growth was present, plugs with uniform growth were then transferred to the experimental plates. A single disc of fungal mycelium was inoculated into 50-mm Petri dishes containing 12 ml of the fresh Fries test solution. Plates were wrapped in Parafilm® and incubated at 22 ºC in the dark with a random distribution within the incubator. Nine plugs of each isolate were immediately harvested and the weight of three plugs taken at random was used to establish the mean dry weight of the mycelia at the start of the experiment.

Mycelia were harvested during the exponential growth phase, in order to avoid lag phases and exhaustion of the medium. In a preliminary experiment, a fast-growing isolate (D44.1) was selected and through its growth curve it was possible to establish an incubation period of 21 days for experimental isolates (Hartley *et al.* 1997). At the pre-determined harvest day, mycelium was collected with a tweezers onto filter paper pads and dried to a constant weight at 50 ºC. The dry weight increase during the experimental period was calculated and expressed as a percentage of the control (also referred as tolerance indices, TI). The effective concentration of Ni inhibiting fungal growth by 50% (EC_{50}) was calculated by fitting the best curves to the data with SigmaPlot v12.0 (Systat Software Inc., San Jose, CA, USA).

A three-way analysis of variance (ANOVA) followed by the Holm-Sidak multiple comparison test was performed to analyze tolerance index. Factors were origin (two levels: serpentine and non-serpentine), ploidy level (two levels: haploid and diploid) and Ni concentrations (seven levels: 0, 0.5, 2, 10, 20, 50, 100 µg g^{-1}). Aneuploid isolates responses were treated as singular events and were thus not compared statistically. A two-way ANOVA was performed to analyze biomass increase at control treatment (no added Ni). ANOVA assumptions were verified before tests were performed. A Mann-Whitney test was performed to compare mean EC_{50} values within soil source and ploidy levels. All the analyses were performed using Sigmaplot v12.

RESULTS

A differential response in plug size was visible over different isolates and Ni concentrations. The three-way ANOVA showed a non-significant interaction between source soil vs. ploidy level vs. nickel concentration (F=0.039, p=0.915; Table 4). Also, soil source vs. ploidy level interaction showed not to be statistically significant (F=0.233, p=0.631). However, there was a significant interaction between soil source and Ni concentration (F=11.360, p<0.001); indeed, the tolerance index between both soil sources showed to be significantly different (F=77.169, p<0.001; Fig. 8).

Table 4 Three-way ANOVA results for soil source, ploidy and nickel as grouping variables

Source of Variation	DF	SS	MS	*F*	*P*
Soil source	1	71587,687	71587,687	77,169	<0.001**
Ploidy level	1	272,981	272,981	0,294	0,589
Ni concentration	6	224003,457	37333,909	40,245	<0.001**
Soil x Ploidy	1	215,795	215,795	0,233	0,631
Soil x Ni	6	63230,145	10538,358	11,360	<0.001**
Ploidy x Ni	6	18448,195	3074,699	3,314	0,005*
Soil x Ploidy x Ni	6	1889,615	314,936	0,339	0,915
Residual	112	103899,478	927,674	-	-
Total	139	483547,354	-	-	-

SS type III sum squares, *DF* degrees of freedom, *MS* means square, *F* f test
*p<0.05; **p<0.001

Although a non-significant difference was found between the two ploidy levels (F=0.294, p=0.589), a significant interaction was found between ploidy level and Ni concentration (F=3.314, p=0.005).

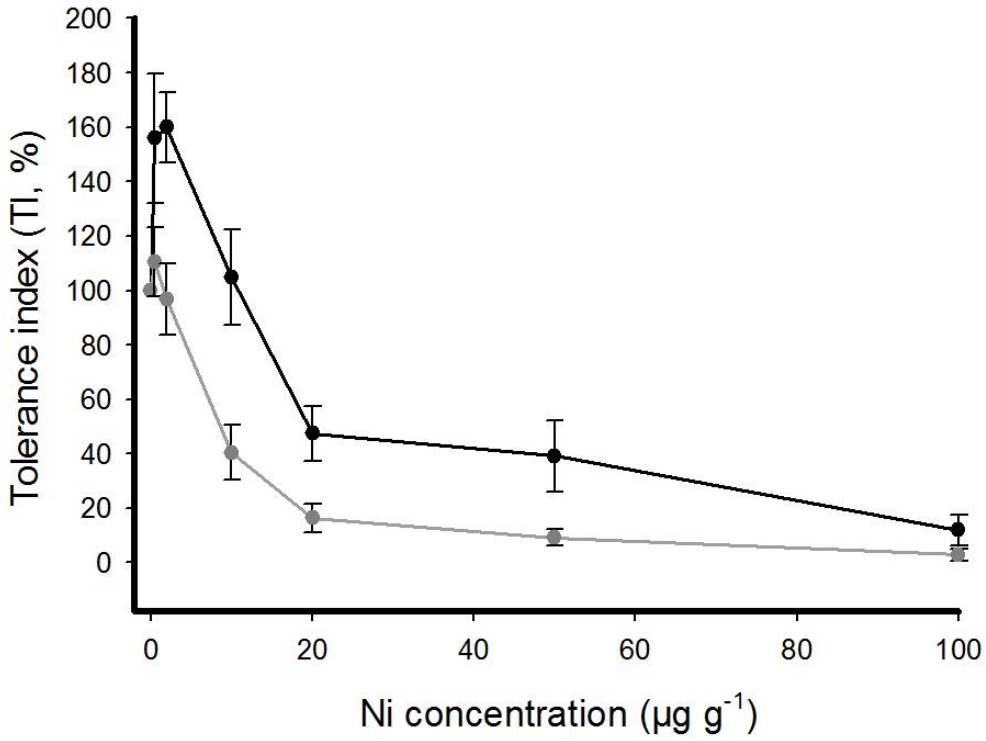

Figure 8 Mean Tolerance index (TI, %) values for serpentine and non-serpentine isolates over a range of Ni concentrations (0.5, 2, 10, 20, 50, and 100 µg g^{-1}).

Different nickel concentrations promoted differences in tolerance index by itself (F=40.245, p<0.001), but also depending on soil source or ploidy level. Along the nickel gradient, differences between both soil sources became slighter. A higher slope means higher difference between soil sources or ploidy levels (Fig. 9).

Differences in tolerance indices were verified between serpentine and non-serpentine isolates, depending on Ni concentrations. At 2, 10, 20 and 50 µg g^{-1} Ni, statistically higher tolerance index values were found in serpentine isolates. Up to 10 µg g^{-1} Ni, the tolerance index was kept above 100% in serpentine isolates, while this percentage was only verified in non-serpentine soils at 0.5 and 2 µg g^{-1} Ni. Similarly to soil source, ploidy level also affected the tolerance index depending on Ni concentration (Fig. 9b). Significant differences were verified at 0.5 (F=3.030, p=0.003) and 10 µg g^{-1} Ni (F=2.904, p=0.004), with higher TI values in haploid and diploid level, respectively.

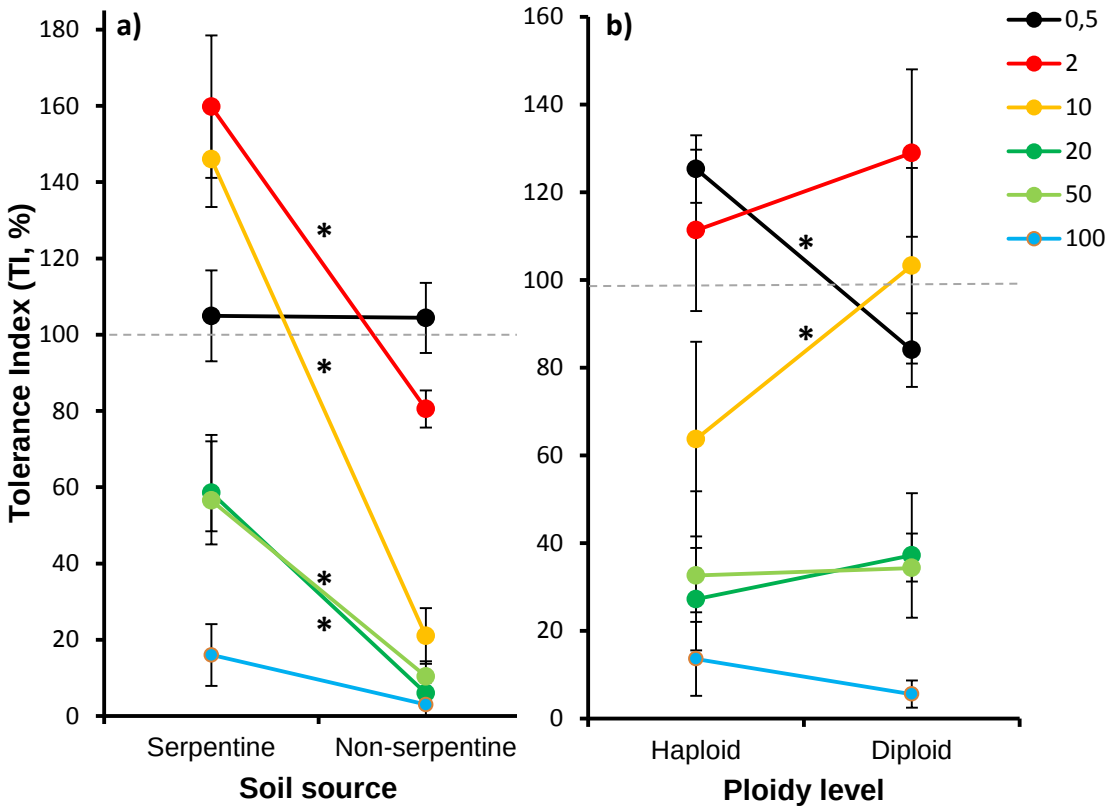

Figure 9 Tolerance index (TI, %) values for **a)** serpentine and non-serpentine isolates and **b)** ploidy level, among nickel concentrations (µg g^{-1} Ni)
***** means statistically significance difference between both soil source or ploidy levels

Estimations of EC_{50} values for each isolate are shown in Appendix B.

Although not significant (p=0.667), EC_{50} was slightly higher in diploid isolates than in haploid ones. Also, non-significant differences between both soil sources were shown (p=0.333), with higher EC_{50} values presented in serpentine isolates. Serpentine diploid A99.1 recorded the highest EC_{50} (52.72 mg g^{-1} Ni) while the lowest value was verified in non-serpentine haploid isolate B44.1 (3.66 mg g^{-1} Ni).

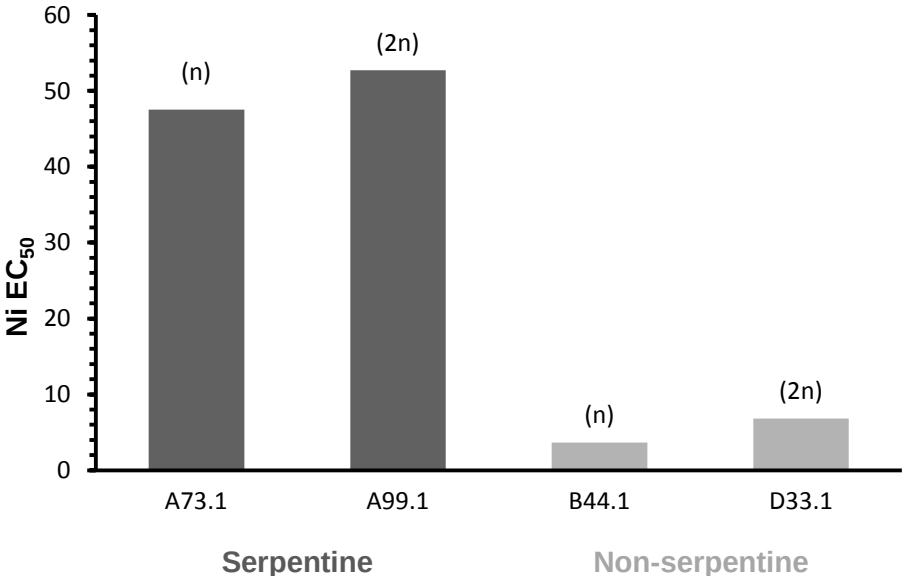

Figure 10 EC_{50} values for all *Cenococcum geophilum* isolates, identified by ploidy level and source soil

Biomass increase was also tested at control treatment (0 µg g^{-1} Ni). Significant differences were found across soil source and ploidy level treatments, but no interaction was found between factors (soil source and ploidy level; Table 5).

Table 5 Two-way ANOVA results for Soil source and Ploidy level as grouping variables

Source of Variation	DF	SS	MS	*F*	*P*
Soil source	1	4620,800	4620,800	22,106	<0.00
Ploidy level	1	1227,222	1227,222	5,871	0,028*
Soil x Ploidy	1	8,889	8,889	0,0425	0,839
Residual	16	3344,400	484,280	-	-
Total	19	9201,311	-	-	-

S
S

type III sum squares, *DF* degrees of freedom, *MS* means square, *F* f test
*p=0.05; **p=0.001

Non-serpentine diploid D33.1 presented the higher biomass increase (68.07 mg g^{-1}), whereas the serpentine haploid A73.1 presented the lowest value (22.00 mg) (Fig. 11). Non-serpentine isolates showed higher values than serpentine isolates, independently of the ploidy level. Diploid isolates showed a higher biomass yield than haploid isolates for both source soils.

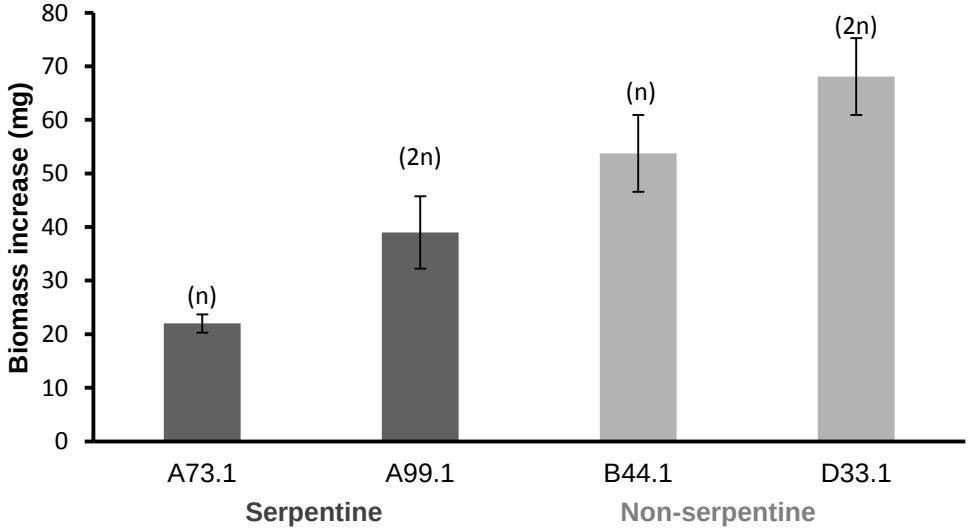

Figure 11 Biomass increase (mg) of haploid (n) and diploid (2n) *Cenococcum geophilum* isolates from serpentine (black bars) and non-serpentine (grey bars) soils when growing in control medium (no Ni added). Each bar is the mean of 5 replicates. Bars represent ± SE of the mean.

Aneuploid isolates showed a distinct behavior from both haploid and diploid isolates. As opposed to haploid and diploid isolates, serpentine aneuploid C6.4 presented a lower EC_{50} value (10.27 mg g^{-1} Ni) than non-serpentine aneuploid B57.3 (17.88 mg g^{-1} Ni). At 0.5 µg g^{-1} Ni the serpentine aneuploid C6.4 recorded the highest TI value of the experiment (257.75%), decreasing dramatically for higher Ni concentrations. Non-serpentine aneuploid B57.3 showed tolerance index values higher than other non-serpentine isolates, reaching up to 50 µg g^{-1} Ni.

DISCUSSION

Isolates of *C. geophilum* from different soil types responded differently to Ni addition: higher Ni tolerance was found in serpentine isolates, according to the Ni status of their soil source. Mean Ni tolerance index was 81.06% and 32.81% in serpentine and non-serpentine isolates, respectively. These results are in agreement with the ones previously obtained by Gonçalves *et al.* (2007, 2009), suggesting that adaptive tolerance to Ni might have evolved in serpentine soils in response to Ni exposure. In fact, these results not only agree, but add to the growing number of *C. geophilum* isolates tested from these two soil types that followed a consistent pattern of response towards Ni exposure *in vitro*.

In this study we took a step further by exploring for the first time the putative effect of the different ploidy levels in the Ni dose-response treatment. Although we did not find significant differentiation in genome size between the *C. geophilum* populations (serpentine and non-serpentine) included in the study (Chapter 1), variations in genome size can still influence the response of individuals to particular environmental features, such as increasing concentrations of Ni (Lidzbarsky *et al.* 2009). According to our predictions, isolates from serpentine sites exhibited lower sensitivity to Ni than isolates from the non-serpentine sites, independently of ploidy level. Still, taking in consideration the haploid and diploid condition in Ni dose-response treatments, dissimilar behaviors were obtained when isolates of each ploidy level were exposed to different nickel concentrations. When comparing haploid with diploid isolates, diploid isolates showed higher tolerance indices than haploid ones for all nickel concentrations except 0.5 μg g^{-1}. This result suggests that diploidization can contribute to adaptation of *C. geophilum* to serpentine soils *via* Ni-enhanced tolerance. In previous studies, similar results were obtained, with an increase in genome size being related to stress adaptation in yeast (Dahr *et al.* 2011, Welker and Williams 1980, Hilton *et al.* 1985, Raffaele and Kamoun 2012).

In control medium (no added nickel) non-serpentine isolates showed higher biomass yield than serpentine isolates (Fig. 11). This vigorous growth of non-serpentine isolates suggests a faster colonization in non-contaminated sites, when compared to serpentine isolates. In fact, a “cost of tolerance" has been

suggested for ECM fungi (Wu 1990, Hartley *et al.* 1997), proposing that adaptation to metal contaminated sites might have a significant cost in terms of energy expenditure when compared to non-adapted ones. Regarding ploidy level, diploid isolates presented a higher biomass yield than haploids at 0 μg g^{-1} Ni, which may confer a competitive advantage in non-contaminated environments. Indeed, a competitive advantage have been suggested for other organisms with multiple sets of chromosomes (Comai L. 2007, Spaniel *et al.* 2008; Hao *et al.* 2013). Once again, a cost of tolerance might explain why, despite being expected a larger number of diploids in non-serpentine populations, haploids were the most represented level of ploidy. A selection for isolates more tolerant to heavy metals might compromise other features important for fungal fitness in non-contaminated habitats. In alternative, it might be that diploid individuals are of recent origin and still did not have time to expand.

Aneuploid isolates responded interestingly (but not unexpectedly) different from each other and from the other isolates. Serpentine aneuploid isolate C6.4, although presenting the lowest EC_{50} value (Fig. 10), showed to be an interesting isolate due to its ability to reach extremely high tolerance index for low Ni concentrations (0.5 and 2 μg g^{-1}), possibly revealing an increased need for this essential nutrient. On the other hand, non-serpentine aneuploid isolate B57.3 kept its TI always above isolates B44.1 and D33.1 TI values, which might be an indication of enhanced tolerance to Ni, which could be explored in bioremediation. According to our estimates, the aneuploids included in this study have a genome size between haploid and diploid isolates. Consequently, it is possible that these isolates originate from a haploid that acquired genetic information, or from a diploid, losing some genetic content (Chapter 1). Therefore, because the basis of aneuploids genetic information is unknown, although interesting, these results should be interpreted with caution.

Overall, this study confirms the suggestion that serpentine soils led to the evolution of Ni tolerance in *C. geophilum*. Additionally, because diploids were more tolerant to Ni than haploids, results from this study suggest a role of genome size in the adaptation of this fungal species to serpentine soils. More isolates should be screened to confirm this result.

Establishing the relationship between soil source, Ni tolerance and genome size variation will help the future identification of the genomic regions involved in *C. geophilum* serpentine adaptation.

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CONCLUSIONS AND PERSPECTIVES

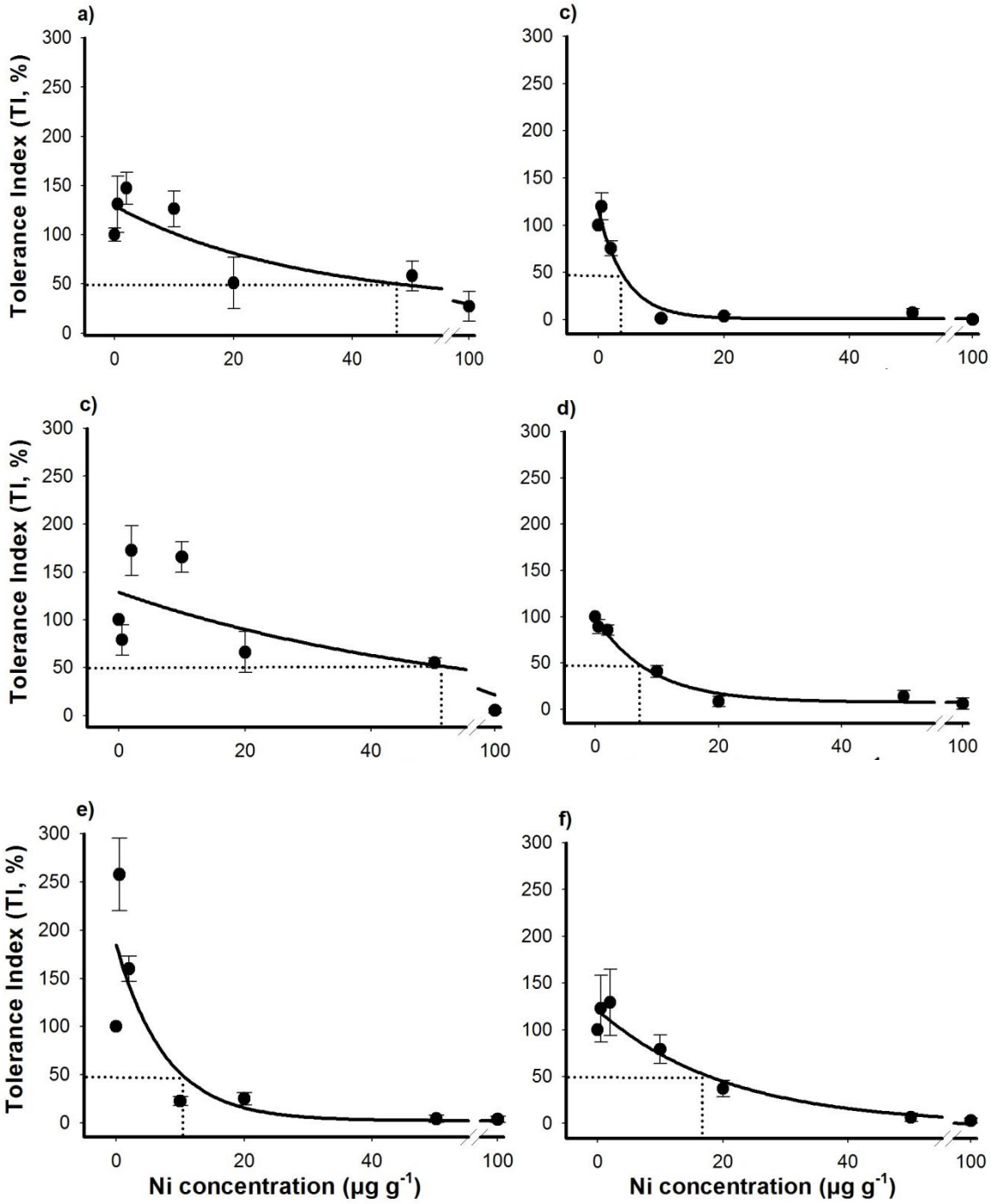
This thesis focused on the determination of *Cenococcum geophilum* genome size and possible relation with its adaptive value in serpentine soils. Forty isolates from serpentine and non-serpentine populations were screened and their genome size estimated. *Cenococcum geophilum* revealed to have one of the largest genome sizes so far measured in the Ascomycota, with a mean haploid genome size estimate of 0.208 pg or 203 Mbp **(Chapter 1)**. Obtained results suggest that variation in ploidy level is not related to serpentine tolerance in *C. geophilum*. The observed variation in genome size, more specifically ploidy level, may however support the existence of a cryptic mating or parasexual cycle. To confirm the presence and/or nature of a cryptic sexual stage, additional analyses are still required in this widely distributed ectomycorrhizal species. The relatively large genome size, providing the potential for local adaptation to develop in vastly different environments, may still contribute to the wide ecological breadth, including the occurrence in serpentine soils, reported for this species.

To verify the possible effect that genome size has in nickel tolerance of serpentine and non-serpentine isolates, six isolates with genome size determined in Chapter 1 were exposed to different Ni concentrations **(Chapter 2)**. Results showed to be in accordance with previous ones, proposing that isolates from serpentine sites will exhibit lower sensitivity to Ni than isolates from the non-serpentine sites. Considering ploidy level, higher tolerance indices were showed by diploid isolates, suggesting that Ni tolerance in diploid *C. geophilum* might ease serpentine adaptation, favoring diploid isolates. Future studies should consider a screening of more isolates from each population, and comprise a higher number of populations. Also, besides Ni concentration, other serpentine stress factors should be explored such as drought and Ca/Mg ratio.

APPENDICES

Appendix A. Genome sizes and ploidy levels of 40 *Cenococcum geophilum* isolates, obtained from two serpentine (S, Espinhosela and Nogueira) and two non-serpentine sites (NS, Rabal and Aveleda) in Portugal, as estimated by propidium iodide flow cytometry; mean values and standard deviations are based on independent triplicate measurements; *mixoploid isolate, SD, standard deviation, CV, coefficient of variation of the *C. geophilum* G0/G1 fluorescence intensity peak.

Population	Isolate ID	DNA ploidy level	Nuclear DNA content		CV (%)
			Mean ± SD (pg)	Mean ± SD (Mb)	
Nogueira	A4.1	haploid	0.223 ± 0.01	219 ± 10	6
	A8.1	haploid	0.248 ± 0.02	243 ± 20	7
	A14.1	haploid	0.197 ± 0.01	193 ± 10	4
	A73.1	haploid	0.208 ± 0.01	203 ± 10	6
	A75.1	haploid	0.191 ± 0.00	187 ± 0	1
	A76.1	haploid	0.224 ± 0.02	219 ± 20	7
	A90.3	haploid	0.221 ± 0.01	216 ± 10	4
	A91.3	haploid	0.240 ± 0.00	235 ± 0	2
	A97.1	haploid	0.207 ± 0.02	203 ± 20	9
	A99.1	diploid	0.410 ± 0.03	401 ± 29	6
Rabal	B5.1	diploid	0.421 ± 0.03	411 ± 29	7
	B22.1	haploid	0.230 ± 0.01	225 ± 10	4
	B26.1	haploid	0.205 ± 0.02	201 ± 20	7
	B35.1	haploid	0.197 ± 0.00	193 ± 0	1
	B44.1	haploid	0.206 ± 0.01	201 ± 10	7
	B48.1	haploid	0.210 ± 0.02	205 ± 20	10
	B49.1	haploid	0.215 ± 0.00	211 ± 0	2
	B51.1	haploid	0.226 ± 0.02	221 ± 20	8
	B57.3	aneuploid	0.361 ± 0.01	353 ± 10	4
	B59.1	haploid	0.217 ± 0.01	212 ± 10	5
Espinhosela	C6.4	aneuploid	0.283 ± 0.02	276 ± 20	6
	C46.2	haploid	0.211 ± 0.01	206 ± 10	3
	C60.1	haploid	0.193 ± 0.00	188 ± 0	1
	C68.1	haploid	0.204 ± 0.01	199 ± 10	3
	C73.1	haploid	0.188 ± 0.01	183 ± 10	3
	C79.2	haploid	0.197 ± 0.02	193 ± 20	8
	C80.1	haploid	0.216 ± 0.00	211 ± 0	1
	C82.2	haploid	0.204 ± 0.02	200 ± 20	3
	C86.8	haploid	0.170 ± 0.01	166 ± 10	6
	C92.1	haploid	0.207 ± 0.00	202 ± 0	1
Aveleda	D3.3	haploid	0.213 ± 0.00	208 ± 0	2
	D22.1	haploid	0.179 ± 0.01	175 ± 10	6
	D32.1	haploid	0.208 ± 0.00	204 ± 0	1
	D33.1	diploid	0.395 ± 0.03	387 ± 29	7
	D35.2	haploid	0.211 ± 0.01	207 ± 10	4
	D38.1	haploid	0.206 ± 0.02	201 ± 20	10
	D44.1	haploid	0.201 ± 0.01	197 ± 10	4
	D47.1	haploid	0.181 ± 0.02	178 ± 20	9
	D67.1	Haploid	0.206 ± 0.01	201 ± 10	7
	D70.1*	diploid	0.439 ± 0.04	430 ± 39	9
Reference	Cg 1.58	haploid	0.246 ± 0.03	241 ± 27	10



Appendix B Tolerance indices (TI, %) of a) A73.1, b) B44.1, c) A99.1, d) D44.1, e) C6.4 and f) B57.3 isolates of *Cenococcum geophilum* studied over a range of Ni concentrations (0.5, 2, 10, 20, 50, and 100 µg g^{-1}). Each point is the mean of five replicates. Bars represent ± SE of the mean. Sigmoid curves were fitted to the data. Line drops indicate the Ni EC_{50} value of each isolate.