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Chestnut blight in Portugal: spread and populational structure of *Cryphonectria parasitica*

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ABSTACT

Cryphonectria parasitica, the causal agent of chestnut blight, has been considered one of the most important mortality factors of the sweet chestnut *Castanea sativa* all over the world. To evaluate the current distribution of chestnut blight in Portugal, all chestnut growing regions across the entire country were screened, a total of 191 chestnut stands were surveyed and *C. parasitica* was isolated from 56.5% of the sampling sites.

The factors involved in the spread and incidence of the disease were studied for one of the most important chestnut areas in the country, located on Trás-os-Montes e Alto Douro. Selected variables were altitude, soil type, diameter at breast height (DBH), location and orientation of cankers in the tree. The highest proportion of canker was found in trees with a DHB above 30 cm, and a higher proportion of cankers was found on the South-facing side of the trunks and branches.

In order to improve the knowledge about *C. parasitica* diversity, a total of 617 isolates from all affected chestnut growing areas were analyzed. Nine vegetative compatibility (vc) types were identified among the isolates. EU-11 was the most widespread vc type comprising 80.2% of the isolates, followed by EU-12 (7.1%) and EU-66 (6.6%). Two of the Portuguese vc types could not be assigned to a known European vc type. The diversity of vc types was low in the Portuguese populations of *C. parasitica*, but comparable to other areas where *C. parasitica* has been recently introduced. The frequent occurrence of perithecia and both mating types of *C. parasitica* indicates that sexual reproduction of the chestnut blight fungus is common in Portugal. One *C. parasitica* isolate from Trás-os-Montes showed white culture morphology and contained dsRNA indicating the presence of hypovirulence in this area.

During this study atypical isolates, strongly related with *C. parasitica*, were found among common isolates. To allocate these unreported isolates to the correct taxon, they were compared to known *C. parasitica* and *C. radicalis* isolates using an integrated approach comprising morphological and molecular methods. Phylogenetic analyses show that these isolates are grouped in a separate clade from *C. radicalis sensu stricto* (phylotype I), but together with other isolates considered as *C. radicalis*, thus allowing their identification as *C. radicalis* phylotype II. Genomic fingerprinting of isolates was performed with microsatellite-primed PCR and diagnostic molecular markers, suitable for the discrimination of *C. radicalis* phylotype I, *C. radicalis* phylotype II and *C. parasitica*, were obtained with $(GACA)_4$ primer.

Key - Words: *Castanea sativa, Cryphonectria radicalis*, hypovirulence, MSP-PCR, genotypic diversity, mating type, RFLP-PCR, vegetative incompatibility

RESUMO

Cryphonectria parasitica (Murrill) Barr é o agente causal da doença conhecida como cancro do castanheiro. Esta doença foi identificada pela primeira vez em castanheiro americano, *Castanea dentata*, nos Estados Unidos da América em 1904 e dizimou quase totalmente os castanheiros americanos nos 40 anos que se seguiram.

A doença foi descoberta na Europa em 1938, embora o fungo tenha sido descrito pela primeira vez em Portugal em 1929 a partir de amostras de castanheiro Japonês, *Castanea crenata*. Depois da detecção inicial, apenas em 1989 foram encontrados dois focos da doença em castanheiro Europeu, *Castanea sativa*, na região de Trás-os-Montes e Alto Douro. Durante algum tempo esta doença ficou associada quase exclusivamente ao nordeste do país.

Na Europa a doença não teve a mesma repercussão que nos Estados Unidos devido ao inesperado aparecimento de fenómenos de hipovirulência transmissível, um fenómeno de controlo biológico natural que, juntamente com a maior tolerância do hospedeiro na Europa, contribuiu para evitar o desaparecimento global dos castanheiros inicialmente sujeitos a fortes surtos de mortalidade. As primeiras formas atípicas do fungo foram isoladas a partir de cancros em regressão, apresentavam virulência reduzida quando inoculadas em castanheiros e, quando co-inoculadas com as estirpes normais, a maioria dos cancros resultantes entrava em compartimentação. O aparecimento da hipovirulência resultou na recuperação de muitos povoamentos de castanheiro europeus.

Os indivíduos portadores do hipovirus transferem o dsRNA viral através de anastomoses hifais, conferindo a outros características de hipovirulência.

A incompatibilidade vegetativa entre indivíduos afecta a transmissão de hipovirulência e, consequentemente, afecta o controlo biológico natural do cancro do castanheiro, sendo a probabilidade de transmissão dos hipovirus uma função do número de diferentes genes de incompatibilidade vegetativa. A incompatibilidade vegetativa é controlada por interacções entre alelos (duas estirpes são incompatíveis quando têm diferentes alelos em um ou mais *vic-loci*), sendo que na Europa é controlada por 6 *vic-loci*, cada um com dois alelos, tendo os 64 genótipos possíveis sido identificados e atribuídos a diferentes tipos de compatibilidade vegetativa (cv).

A reduzida diversidade em grupos cv parece estar directamente relacionada com o potencial de sucesso dos programas de controlo biológico. Uma vez que a transmissão se faz mais rapidamente entre estirpes do mesmo tipo, a introdução de estirpes hipovirulentas só deve ser feita após conhecimento profundo da diversidade de grupos cv existentes em cada local, de forma a não comprometer o sucesso das acções de controlo biológico.

Por outro lado, a transmissão vertical destes hipovirus está limitada a conídios, esporos de produção assexuada, não ocorrendo em ascósporos. Este facto, associado à geração de novos tipos cv por recombinação meiótica, torna a reprodução sexuada um dos maiores obstáculos à dispersão da hipovirulência. Tendo *C. parasitica* um sistema misto de cruzamento, com auto-fertilização e heterotalismo com dois tipos conjugais (MAT-1 e MAT-2), a análise das proporções destes e a avaliação da diversidade genómica permite identificar os mecanismos de reprodução prevalentes (assexuada *versus* sexuada) e caracterizar a estrutura das populações de *C. parasitica*, fornecendo informações essenciais para a planificação de estratégias de luta biológica.

Com este trabalho pretendeu-se em primeiro lugar fazer um reconhecimento da distribuição actual do cancro do castanheiro em Portugal (continente e Regiões

Autónomas da Madeira e Açores), tendo sido com esse objectivo implementado um trabalho de prospecção a nível nacional. Foram prospectadas 191 parcelas de castanheiro em todo o país e verificou-se a presença de *C. parasitica* em 56,5% das amostras (108 parcelas). As parcelas amostradas foram digitalmente georeferenciadas e cartografadas. Uma colecção de isolados de *C. parasitica* foi estabelecida, constituindo uma base uma base de trabalho para os estudos posteriores.

Com o objectivo de avaliar os factores que poderiam estar envolvidos na dispersão e incidência da doença, foi escolhida a Região Demarcada da Padrela em Trás-os-Montes, por ser uma zona do país importante do ponto de vista de área de castanheiro e produção de castanha e onde se verificavam muitos registos da ocorrência da doença. Na área de estudo foram prospectadas 32 parcelas, distribuídas por 4 freguesias do concelho de Valpaços, e elaboradas fichas de caracterização geral para cada parcela e árvores observadas. Foram analisadas variáveis associadas à parcela (altitude, tipo de solo, orientação) e às árvores (localização predominante dos cancros, orientação predominante dos cancros, diâmetro à altura do peito - DAP).

A incidência da doença foi estimada em 40% para a área de estudo, verificando-se uma maior incidência de cancro nos ramos e nas árvores cujo DAP era superior a 30 cm, havendo uma tendência para uma maior predominância de cancros do lado sul da árvore. Não se verificou correlação entre os restantes factores analisados e o aparecimento de cancro na zona de estudo.

Para avaliação da diversidade genética de *C. parasitica* foram analisados 617 isolados provenientes de todas as regiões do país. Foram efectuadas confrontações entre toda as culturas recolhidas, tendo-se encontrado nove tipos diferentes de compatibilidade vegetativa. Culturas representantes de cada um dos nove tipos cv foram posteriormente confrontadas com culturas representativas dos tipos já estabelecidos na Europa.

Concluiu-se que o tipo cv mais frequente em Portugal é o tipo europeu EU-11 (80,2% dos isolados testados pertenciam a este tipo), estando este amplamente disseminado por todo o país. O tipo cv europeu EU-12 foi encontrado em 7,1% dos isolados portugueses e o tipo cv EU-66 em 6,6%. Dois dos nove tipos cv encontrados não mostraram compatibilidade com nenhum dos Europeus, pelo que se consideram ser dois novos tipos. Os resultados deste trabalho permitem assim afirmar que temos no país uma diversidade genética ainda baixa (índice de diversidade variando entre 0,146 e 1,209), comparativamente com outros países da Europa onde a doença já se estabeleceu há mais tempo (França, Suiça, Itália), sendo os valores comparáveis com os dos países onde a introdução da doença ainda é recente (Eslováquia, Macedónia, Turquia, Alemanha).

Através da projecção que foi efectuada com base nos tipos cv encontrados em Portugal, constatou-se a existência de um potencial genético que promoverá uma maior diversidade genética no futuro. De facto, assumindo a recombinação dos *vic loci* conhecidos, o número de tipos cv poderá atingir 32 e será certamente mais elevado se houver introdução de outros tipos cv através de entrada no país de material vegetal contaminado. A ocorrência de peritecas (estruturas de frutificação sexuada) em muitas regiões, assim como a existência dos dois tipos conjugais (MAT-1 e MAT-2) com proporções idênticas, indica que a reprodução sexual do fungo é um fenómeno frequente em Portugal.

Foi confirmada a presença de dsRNA viral num isolado, proveniente de Trás-os-Montes.

A associação da baixa diversidade vegetativa, encontrada actualmente no país, com a existência de hipovirulência natural será um factor importante para o controlo da

doença em Portugal. Sendo o dsRNA viral mais facilmente transmitido entre indivíduos pertencentes ao mesmo tipo cv, o facto do isolado hipovirulento pertencer ao tipo mais comum (EU-11) faz prever que estarão reunidas condições para uma dispersão facilitada da hipovirulência. Porém, se a baixa diversidade é um factor positivo, por outro lado a evidência de uma alta incidência de reprodução sexuada em Portugal é um factor negativo, uma vez que os ascósporos não permitem a transmissão do hipovirus e a recombinação sexual aumentará a diversidade genética.

Com o conhecimento da distribuição da doença e depois dos estudos genéticos efectuados no âmbito deste trabalho, está disponibilizado conhecimento que faculta o entendimento da dinâmica da doença em Portugal e constitui a base necessária para a implementação segura de programas experimentais de luta biológica no nosso país.

Durante este estudo obtiveram-se ainda culturas atípicas entre os isolados de *C. parasitica*, verificando-se posteriormente que esses isolados revelavam as mesmas características morfológicas de isolados anteriormente recolhidos em sobreiro e classificados como *Endothiella gyrosa*. Para a sua correcta identificação, estes isolados foram comparados com isolados de *C. parasitica* e de *C. radicalis*, sendo esta uma espécie do mesmo género que partilha os mesmos hospedeiros, mas que é considerada saprófita. Para a identificação foram utilizados de uma forma integrada métodos morfológicos e moleculares, que permitiram identificar estes isolados como pertencendo à espécie *C. radicalis* filotipo II. As análises filogenéticas separaram este filotipo II do filotipo I, considerado por alguns autores como *C. radicalis sensu stricto*. Verificou-se com a técnica de MSP-PCR uma clara separação dos três grupos analisados, *C. parasitica*, *C. radicalis* filotipo I e *C. radicalis* filotipo II, tendo sido definidos marcadores moleculares, através da utilização do "primer" $(GACA)_{4,}$, para distinguir de uma forma rápida isolados pertencentes a estes grupos de fungos.

PART I

GENERAL INTRODUCTION

REVIEW OF CHESTNUT TREE – ORIGIN, DISTRIBUTION, SOCIO-ECONOMIC AND ENVIRONMENTAL IMPACT

The chestnut tree (*Castanea*: Fagaceae) is recognized as one of the most remarkable trees in the world due to its economical and anthropogenic importance. Chestnuts belong to the genus *Castanea*, in the Fagaceae family, together with other important tree species, such as oaks (*Quercus* sp.). For centuries, species within the genus *Castanea* have been a primary nutritional resource for native communities in many areas of Asia, Southern Europe and most countries bordering the Mediterranean Sea. In North America, chestnut was a dominant component of the broadleaf forest in the Eastern United States, until chestnut blight caused by *Cryphonectria parasitica* (Murril) Barr, literally eradicated the species from this region of the world (ANAGNOSTAKIS, 1987).

Historical and anthropological studies have shown a strong interaction between man and chestnut. Evidence exists for the presence of chestnut in prehistoric ages, providing nourishment and fuel (fruits and wood) for the subsistence of the first prehistoric men (ADUA, 1999). It is believed that chestnut was already present in Europe during the final period of the Mesozoic Era, widely spreading approximately 65 million years ago, during the Cenozoic Era (MAIA, 1988; ADUA, 1999). During the glaciations (Quaternary Era), chestnut persisted in the Caucasian-Armenian area and it was in this Asian region, in the IX-VIIth century B. C. that chestnut cultivation began and quickly spread from Asia Minor to Greece and the Balkanic region (ADUA, 1999). According to VILLANI *et al.* (1999) chestnut also survived in the Iberian peninsula and Italy during the glaciations.

The Greeks advanced the cultivation of *C. sativa* and during the *Magna Graecia* period, plants and fruits reached Southern Italy. Soon, the Romans discovered the large potential of the chestnut tree, developing techniques to selected new varieties and ultimately disseminate chestnut cultivation from Italy into France, Spain, Portugal, Switzerland and Britain (ADUA, 1999; ABREU, 2007)

During the middle Ages, cultivation and preservation techniques improved and chestnut trees became an important component in the diet of local human populations and to manor economy. Even when cereal production gained more importance, the "bread of the poor", primarily based on chestnut nut flour, remained a basic element in the diet of many people - which still prevails - in under-developed regions of the Iberian

Peninsula, Central France, Central and Northern Italy, Switzerland and in the Balkanic regions (ADUA, 1999; HEINIGER 2001; ABREU, 2007). Starting in the beginning of the XIXth century, up to the first quarter of the XXth century, diseases, deforestation, human migration into cities, increase of synthetic tannin industry, war and socio-economic changes relegated chestnut to a secondary role in modern agriculture, leading to a decrease in chestnut area (ADUA, 1999). However, in some regions and in the last quarter of the XXth century, a progressive revaluation of natural resources according to new directions in sustainable agriculture policies throughout the world, recognized the importance of chestnut, and new chestnut management practices, both for timber and nut production, followed these new premises in order to enhance the vitality of the chestnut stands (BOUNOUS, 2005).

In Western Europe, nowadays, chestnut is no longer critical for subsistence but it still plays a significant role in timber markets and landscape strategies in many agro-forested systems. A detailed description of the multifunctional role of chestnut and the efforts done to improve the technologies and knowledge in the sustainable management of this natural resource has been made by BOUNOUS (2005).

The natural distribution of the chestnut includes three major geographical areas in the world: Asia, with *Castanea crenata* Sieb. & Zucc (in Japan), *C. molissima* Blume. (in China and Korea), *C. seguinii* Dode, *C. davidii* and *C. henryi* Rehder and Wilson (Skan) (in China); North America, original distribution area of *Castanea dentata* [Marsh.] Borkh, *C. pumila* (L.) Mill., *C. floridana* Ashe (Sarg.) and *C. ashei* (Sudw.) Ashe, *C. alnifolia* Nutt., *C. ozarkensis* Ashe and *C. paucispina* Ashe; and Europe with sweet chestnut (*Castanea sativa* Mill.) (ANAGNOSTAKIS, 2005; BOUNOUS, 2005). In Europe, *C. sativa* is distributed throughout the countries in the Mediterranean basin (mainly in the Northern part) but it is also present in Northern Africa, Southern Great Britain and the Caucasus.

Many countries have potential areas where soil and climatic conditions are suitable for the cultivation of chestnut and new orchards of both Asian and European species have been recently established (BOUNOUS, 2005). China and South Korea have increased their chestnut productions and, at present time, are the world's leading producers, with a solid export market and a vast number of new orchards. In Western Europe the main producers are Italy, Turkey, Portugal, Spain, Russian Federation, France and Greece (BOUNOUS, 2005). The Portuguese Agriculture Bureau (Ministerio da Agricultura, 2007) has released the data compiled by the Food and Agriculture

Organization (FAO) for the worldwide production of chestnut fruit, which is currently estimated in 1.1 million T, distributed in a relatively scarce area of 340 000 ha. China is the largest producer accounting for an annual volume of 800 000 T (approx. 70% of the global production). Europe accounts for 12% of the global chestnut fruit production. Of these, 10% are produced within European Union countries, with Portugal and Italy generating 4 and 3% of the worldwide production, respectively.

CASTANEA SATIVA IN PORTUGAL

In Portugal, analysis of fossilized chestnut pollen with an origin on Miocene era evidences the occurrence of chestnut (TEIXEIRA and PAIS, 1976; VAN DEN BRINK and JANSSEN, 1985), in parallel to other reports from central Europe (CONEDERA and HEINIGER, 1994). However, phyto-sociological studies support the assumption that the Romans were the ones to establish chestnut in its cultivated form in Portugal (MALATO-BELIZ, 1987).

In the Portuguese mainland, the chestnut occurs predominantly at North of parallel 39^{o}, generally in zones between 400 *m.s.m.* and 1 000 *m.s.m.* altitude levels and at Sea level in Azores ("Região Autónoma dos Açores") and Madeira ("Região Autónoma da Madeira") Islands, with broader areas distributed in Trás-os-Montes e Alto Douro and Beira Interior (MARQUES, 1988). Trás-os-Montes e Alto Douro is the most important chestnut-growing region in Portugal with approx. 85% of the total area of chestnut in the country, followed by Beira Interior, the second most important area, with approx. 9% in area and production (MINISTÉRIO DA AGRICULTURA, 2007).

Chestnut is an important species for the economy of rural communities due to its dual utility as a fruit and timber producer. Chestnut timber presents good quality, elasticity and an average hardness. It is also an easy wood to work with and resilient to moisture. Due to these properties chestnut wood has been, and still is, broadly used in cooperage, construction and furniture industries. At present time, the majority of chestnut orchards are specifically directed to the production of fruit. The nut production remains an excellent cultural option in many regions of the country, and the Portuguese varieties are recognized in the international markets for their high quality.

An accurate estimate of the current area of chestnut in Portugal is difficult. The area associated with chestnut nut production is approx. 30 x 10^3 ha and corresponds to

orchard areas at full production capacity, which are monitored by the different regional agriculture bureaus (INE, 2007). Provisional data from the last forest inventory of the new National Forest Inventory (INE, 2007) provide an estimated total area of chestnut stands of approx. 29.2 x 10^3 ha (Table 1). This area is supposed to represent all stands, not only orchards, thus it would be expected that this area be higher than the one for fruit production, which is not the case. When comparing data from INE (2007) related to 1995 and 2005 (Table 1) we observed a significant decrease in total chestnut area, from 41.4 x 10^3 ha to 29.2 x 10^3 ha. However, analyzing similar data from the Agriculture Bureau for the period of 1999-2004 (MINISTÉRIO DA AGRICULTURA, 2007), the area restricted to orchard chestnut stands does not show a similar pattern. In opposition, there is an increase of orchard area in Minho e Trás-os-Montes e Alto Douro which is incongruent with the data presented in the Forest Inventory for the same region ("Norte" in Table 1).

We believe that although a decrease in area may have occurred due to biotic or abiotic effects, such as pathogenic diseases or fires, differences are not as significant as the reported by the National Forest Inventory. These differences could be connected with used inventory methodologies. In summary, for the National Forest Inventory, aerial photography is used to classify a pre-determined set of geographic points. The area associated with a specific species is estimated as the ratio between the proportion of points for that species and the area of the country. This methodology is biased, since two errors occur: the limitations in area computation and the species classification error. The error due to computation of the species area will increase in direct proportion with the decrease in the number of sampled points. Based on this premise and since the area of chestnut if relatively small, the error due to area computation is therefore larger. This error has been estimated in 6% for pure chestnut stands, and higher in mix stands (M. TOMÉ, 2007 - pers. comm.). This estimate corresponds to years where the number of sampled points was raised in comparison with the previous decade, 1995, and thus it is reasonable to believe that the error is now minor then it was in the last decades. In addition to the difficulties mentioned above with the methodology, there is also the difficulty inherent to the subjective human analysis of the aerial photography. The interpretation is more difficult for species with a scarce representation such as chestnut or when high forest canopies or coppice are abundant. The season when the photographic material is collected and/or the difficulty to distinguish chestnut stands

from oak-groves also accounts for an increase in the difficulty in photographic interpretation.

Table 1 – Chestnut distribution in Portugal by NUT II division
(data from DGRF National Forest Inventory, INE 2007)

NUT II [a]	1995	2005
	chestnut (10^3ha)	
Norte	33.7	24.5
Centro	6.2	3.2
Lisboa e Vale do Tejo	0.2	0.0
Alentejo	0.1	0.5
Algarve	0.2	0.0
Açores[b]	0.0	0.0
Madeira[b]	1.0	1.0
Total	41.4	29.2

[a] Map in Annex I [b] Estimated value

The chestnut fruit production in 2006 reached 30 886 T, with an average productivity of 0.74 T/ha (Table 2), accounting for more than half (64%) of the national production of dry fruits and 5.4% of the global Portuguese production of fruits (INE 2007). The average Portuguese productivity of adult chestnut stands averages 1 T/ha, a number drastically lower than the ones reported by FAO in 2005 for European countries with chestnut fruit industry, such as Italy (21.6 T/ha), France (18.5 T/ha), Greece (15.7 T/ha) and Spain (16.6 T/ha) (MINISTÉRIO DA AGRICULTURA, 2007). Nonetheless, it is worth mentioning that chestnut stands planted in recent years have followed well-structured silviculture and sustainable management plans, which may lead to an increase in fruit production similar to the one in countries such as the mentioned above.

Table 2 – Orchard area and chestnut fruit production in Portugal (INE 2007)

	Surface (ha)			Fruit Production (T)		
Years	2004	2005	2006	2004	2005	2006
Total values	30 227	30 265	30 253	31 051	22 482	30 886

For the chestnut seedling production in nurseries, intended for the establishment of new chestnut plantations, a significant increase has been recorded, with the region of Trás-os-Montes e Alto Douro presenting the highest productivity. Trás-os-Montes e Alto Douro is the most important region for this plant species, with a reported increase of 2 680 chestnut trees from 2004 to 2006 (INE, 2007). In parallel to an increase in seedling production, a relevant increase in the number of imported chestnut seedlings has been also recorded (Table 3).

Table 3 – Trees sold in nursery-gardens by Agricultural Departments in Portugal (INE 2007)

Agricultural Departments [a]	2004/2005	2005/2006	Increase
	Nº of trees		
Mainland	**91 900**	**95 138**	**3238**
Entre Douro e Minho	8 662	9 445	783
Trás-os-Montes e Alto Douro	58 142	60 822	2680
Beira Litoral	9 356	9 662	306
Beira Interior	9 678	8 998	-680
Ribatejo e Oeste	4 323	4 417	94
Alentejo	1 486	1 598	112
Algarve	253	196	-57
Imported trees [b]	**137**	**1 311**	**1174**

[a] Map in Annex II**.** [b] Trees sold directly to the agriculture and which do not account for the total number of trees.

New chestnut plantations have been promoted by government projects aiming to encourage the reforestation of agricultural lands (European Economic Community -EEC programs: PAMAF; council regulation n.º 2080/92 and RURIS). According to the IFADAP institute – "Instituto de Financiamento e Apoio ao Desenvolvimento da Agricultura e Pescas" new chestnut stands have been planted in compliance with EEC council regulation n.º 2080/92 program, which allowed establishing throughout the country 2 841 new chestnut stands, corresponding to 7 265.95 ha (5 305.45 ha in Trás-os-Montes e Alto Douro) for the period ranging from 1995 to 2000 (Table 4). Data from RURIS were not available.

Table 4 – New projects (R 2080/92) initiated in Portugal between 1995 and 2000

Agricultural Departments	Projects	Area (ha)
Entre Douro e Minho	214	428.87
Trás-os-Montes e Alto Douro	919	5 305.45
Beira Litoral	135	179.35
Beira Interior	335	1 282.47
Ribatejo e Oeste	25	61.47
Alentejo	3	7.70
Algarve	1	0.60
Total	**1 632**	**7 265. 91**

Due to its renowned quality, the Portuguese chestnut fruit has a relevant position in international markets producing a sound commercial advantage translated in economical outcomes, with a balance of 12.6 million Euros, in average, for 2000-2004.

The main market for Portuguese chestnut nut is Spain, as well as, markets with Portuguese immigrated communities such as Brazil, France and Switzerland among others (MINISTÉRIO DA AGRICULTURA, 2007). In addition, owing to increasing market demands for high quality products and the ecological concerns to preserve a national genetic heritage, the creation of stake regions with Protected Denomination of Origin (PDO) has become critical for the value recognition of the regional nut varieties. The use of PDO entails the name of a region, a specific locality or in exceptional cases a country, allowing referencing an agricultural product or other dietary product. PDO requires that the specific product (chestnut fruit in this case) is produced in accordance with regulations prescribed in the specification booklet.

There are four PDO for chestnut fruit in Portugal (Annex III): "Castanha da Terra Fria", "Castanha da Padrela", "Castanha dos Soutos da Lapa" and "Castanha do Marvão" (MANTAS *et al*., 1994; COSTA *et al.* 2005; OLIVEIRA, 2005; FERREIRA-CARDOSO PIMENTEL-PEREIRA, 2007).

- The "Castanha da Terra Fria" (12 500 ha) is produced in the counties of Alfândega da Fé, Bragança, Chaves, Macedo de Cavaleiros, Mirandela, Valpaços, Vimioso and Vinhais and the base production is the variety 'Longal' (more than 70% of the production). These nuts are characterized by a brownish red coloration, with dark longitudinal threads and an elliptical elongated shape.
- The "Castanha da Padrela" (4 600 ha) groups the nuts of the 'Judia', 'Lada', 'Negral', 'Cota', 'Longal' and 'Preta' varieties, which are well adapted to the region where they are produced. The geographic region for its production covers some localities of Chaves, Murça, Valpaços and Vila Pouca de Aguiar counties.
- The "Castanha dos Soutos da Lapa" (4 000 ha) includes the 'Martainha' and 'Longal' varieties. The 'Martainha' has a clear brown color and spheric shape. Its geographic area is circumscribed to localities within the counties of Armamar, Tarouca, Tabuaço, S. João da Pesqueira, Moimenta da Beira, Sernancelhe, Penedono, Lamego, Aguiar da Beira e Trancoso.
- The "Castanha do Marvão" (550 ha) comprises nuts of the 'Bária' variety (dark dull brown), 'Colarinha' or 'Enxerta' varieties (bright brown) as well as

'Bravo' (bright redish). Its geographic region is restricted to the Marvão, Castelo de Vide e Portalegre counties.

Concerning the net value of the main nut varieties, the evaluation of the market value stock rate according to the Agriculture Bureau (Ministério da Agricultura, 2007), for the period 2000-2005 reveals that the cultivar 'Judia' presents the maximum market value and that devaluation of both 'Cota' and 'Longal' varieties has occurred, with oscillations through this period, and which were a function of national and international market size and demands. However, these two varieties have been developing a solid position in international markets, namely in France and Italy (MINISTÉRIO DA AGRICULTURA, 2007).

C. SATIVA IN MADEIRA AND AZORES ISLANDS

In the Portuguese archipelago of Madeira ("Região Autónoma da Madeira"), chestnut was initially introduced in the XVIth century, following the first human settlements, in order to halt hill slope erosion. The current distribution of chestnut in the archipelago is mainly restricted to mountainous zones along the Southern cost of the Madeira Island, usually in plots presenting very irregular or uneven terrain with harsh climatic conditions and where other type of tree or crop would strive. At present, chestnut occupies an area of 71 ha, distributed in approximately 392 holdings, with an estimated nut production of 700 T (of which only 30% are collected). The distribution is mainly in the Câmara de Lobos county (43%), with special emphasis for the Curral das Freiras, where the chestnut fruit has traditionally a higher importance; Ribeira Brava (48%); Funchal (4%) e São Vicente (2%). The varieties with the highest organoleptic importance are the "Negrinha", "Formosa" and "Do tarde." (MAIA, 2003).

In the Azores archipelago ("Região Autónoma dos Açores"), the chestnut is cultivated in the coastline, between 100 and 200 m of altitude, in wind sheltered spots with deep soil and good permeability. Chestnut stands dedicated to nut production occupy approx. 100 ha (see Table 5), with 65% of the total nut production located in the Terceira Island, mainly in Terra-Chã county (comprising the majority of sweet chestnut stands - approx. 80 orchards) and also in Posto Santo, São Pedro and Biscoitos counties (FERNANDES, 1987; ORMONDE, 1994). The predominant variety in Terceira is 'Viana'.

There are chestnut orchards in Pico and Graciosa Islands, but in the remaining Islands chestnut is distributed with a dispersed pattern, with few exceptions.

Table 5 - Chestnut area (SREA, 2003) and chestnut fruit production in Azores ("Região Autónoma dos Açores") (SREA, 2005)

	Surface (ha)	**Fruit Production (T)**
Terceira	70.7	187.1
S. Miguel	17.0	45.4
Pico	10.3	37
Others	4	11.2
Total	102.0	280.7

In contrast to the Madeira archipelago, there is no consensus regarding the introduction of chestnut into the Azores due to the fact that, in some localities, it is considered to be of natural spontaneous growth (FERNANDES, 1987). Some authors consider that the species has been present from the beginning of the human colonization of these areas since there are references to this tree in "Saudades da Terra", written by Gaspar Frutuoso in 1590 (FERNANDES, 1987; ORMONDE, 1994).

DISEASES AND INSECT PESTS OCCURRING ON CHESTNUT

The most notable and studied chestnut diseases are ink disease (root disease), attributed to oomycete species of *Phytophthora*, and chestnut blight (canker disease), caused by the ascomycete fungus *Cryphonectria parasitica* (MACDONALD, 1993).

Different phytopathogens can affect chestnuts worldwide, whether in natural or in cultivated settings, causing damage or death. These diseases are grouped by foliar pathogens, canker induced pathogens and root rots. Fruit damages can also occur and be caused by fungi. Foliar diseases of chestnut, like those of many other deciduous broadleaves, are traditionally believed to be of little importance despite of their negative effects on the photosynthetic activity. However, in specific cases, these diseases could have a great impact. Numerous fungi have been reported to colonize chestnut leaves (categorized as leaf spots, powdery mildews and rusts). The most commonly reported leaf spots of chestnut include those caused by the fungi: *Mycosphaerella punctiformis* (Pers.: Fr.) Starb.; *Mycosphaerella maculiformis* (Pres.) Schroet; D*iscella* (= *Marssonina*) *ochroleuca* (Berk. & M. A. Carits) Arx.; *Tubakia* (= *actinopelte*) *dryina*

(Sacc.) Sutton; *Monochaeta monochaeta* (desmaz.) Allesch. In Rabenh.; and the bacterium, *Pseudomonas castanea* Kawamara. The powdery mildews of chestnut include those caused by *Microsphaeria penincillata* (Walls.:Fr.) Lév. and *Phyllactinia guttata* (Walls.:Fr.) Lév.. Occasionaly, uredia and telia of the rust fungus *Cronartium quercuum* (Berk.) Miyabe ex Shirai appear in abundance on the under surface of chestnut leaves (MACDONALD, 1993).

Canker fungi parasite the bark tissues of the host, including phloem and cambium, and they frequently invade sapwood tissues of the xylem. As a result, small necrotic lesions or large stem deformities (exposing xylem tissues) can be observed. *Cryphonectria parasitica* (Murr.) Barr. is the most destructive canker disease of chestnut. *Endothia gyrosa* (Schwein. :Fr.)Fr.; *Amphiporthe* (=*Cryptodiaporthe*) *castanae* (Tul.) Barr.; *Botryosphaeria* spp.; *Melanconium cinctum* Berk. & M. A. Curtis, *Nectria* spp.; *Cytospora* spp. and *Coryneum modonium* (Sacc.) Griffon & Maubl. have also been reported as canker agents (MACDONALD 1993; ABREU, 1996).

Ink disease (caused mainly by *Phytophthora cambivora* (Petri) and *Phytophthora cinnamomi* Rands species), *Armillaria* root rot (caused by species of *Armillaria*) and Dematophora root rot (*Rosellinia necatrix* Prill) are the most known root diseases of chestnut. Ink disease pathogens cause brownish-black lesions on the roots that exude an ink-blue stain, hence the name ink disease. Trees die when the root collar is girdled, or when most of the roots are killed (FONSECA *et al.*, 2004). *Armillaria* spp., in the Basidiomycetous fungi division, produces honey-colored mushrooms and causes root decay by degrading the lignocellulosic biomass of the wood. It affects chestnut mainly in cases where trees are stressed (by biotic or environmental stress) (KILE *et al.* 1991; GUILLAUMIN *et al.*, 2005). *R. necatrix* is an ascomycetous fungus which causes a white root rot of chestnut and appears to be favored by relatively cool, moist soils.

Chestnut seeds can be infected by a variety of fungal spores and bacteria which can occur mainly during storage: *Ciboria batschiana* (Zopf.) Buchwald (the most important fruit pathogen); *Phomopsis endogena* (Speg) Cifferi; *Phomopsis castanea* (Sacc.) Höhn; *Penicillium* spp.; *Mucor* spp.; *Acrospeira mirabilis* Berk.; *Trichothecium roseum* (Pers.) Link.; *Fusarium oxisporum* Schiltdl; *Wardomyces columbinus* (Demelius) Henneberg; *Clonostachys rosea* (Link) Schoers, Samuels, Seifert and W. Gams and *Anphiporthe castanea* (Tul. and C. Tul.) (BISSEGER and SIEBER 1994; CONEDERA *et al.* 2004).

Until recently, it was considered that the most problematic insects associated with the chestnut tree attack the fruit and include the species *Pammene fasciana* L., *Cydia fagiglandana* Z., *Cydia splendana* (Hb) and *Balaninus (Curculio) elephas* Gyll (BREISCH 1995; CONEDERA *et al.* 2004; DELISLE *et al.* 2005). In addition, *Xylebore dispare* F., an insect that attacks the trunk and branches, has been at present time frequently found on chestnut. Moreover, the bark miner *Spulerina simploniella* (Lepidoptera: Gracilariidae) has been also found in coppice chestnut (*C. sativa*) forests. Its larvae mine under the thin periderm of young trees, 4–10 years old, while the stem bark is still smooth. Under normal conditions it does not cause any damage to the trees. However, when chestnut blight is present in the area, the insect may be an agent of disease spread (DIAMANDIS and PERLEROU, 2005).

At present time, *Dryocosmus kuriphilus* Yasumatsu, the Oriental chestnut Gall Wasp, is considered as the most serious pest of chestnut worldwide. Known in the U. S. since 1974, it was imported from Asia to the Eastern Georgia (PAYNE *et al.* 1976) and has recently been introduced in Europe (Piemonte, Italy) (BOSIO *et al.*, 2003). The EPPO Panel on Phytosanitary Measures considered that this species should be added to the EPPO Alert List. Spread of the pest from the infested area in Southern Piemonte is very likely to occur through flying females and the movement of infested young chestnut plants and grafts (BOSIO *et al.*, 2003). Females lays eggs in the leaves and flower buds and, as the larvae develop, green or rose-colored galls are formed on the leaves, petioles, and even catkins. These galls shoot elongation and reduce fruiting, and trees with severe infestations lose their vigor and often die (ANAGNOSTAKIS 1999, 2001).

REPORTS FROM PORTUGAL

The reduction of the Portuguese chestnut area has been attributed to socio-economic changes and also to the occurrence of pathogenic fungi such as *Phytophthora* spp. and, in the last two decades, *C. parasitica*.

References to the occurrence of ink disease (causal agent *Phytophthora* spp.) in Portugal (Minho province) date from the XIXth Century (FERNANDES, 1966; ABREU, 1995; MARTINS and ABREU, 2007), although only in 1942 the presence of *P. cinnamomi* e *P. cambivora* was confirmed in diseased chestnut tissue (PIMENTEL, 1947). This

aggressive root pathogen is at present widely distributed across Portugal, occurring on other hosts as well, especially cork oaks. Although *P. cambivora* has been occasionally isolated from chestnut tissues in the past, *P. cinnamomi* is by far the most prevalent species in chestnut orchards and nurseries in the Northeastern Portugal (MARTINS *et al.* 1999; FONSECA *et al.* 2004). Diffuse cankers caused by *Melanconis modonia* Tul. (anamorph *Coryneum modonium* (Sacc.) Griffon & Maubl.) have been recorded in Portugal on senescent bark or chestnut weakened by biotic and abiotic factors. In 1995 this fungus was reported as a canker agent on chestnut tree causing significant losses (ABREU, 1996; ABREU and GOMES, 1989). *Mycosphaerella maculiformis* was also reported by ABREU and GOMES (1989), affecting adult chestnut trees in Northeast of Portugal.

Concerning insect pests, references to *Melolontha* sp. affecting root plants on nurseries and *Zeuzera pyrina* L. and *Xyleborus dispar* F. (Coleoptera) found on trunks of chestnut adult trees exist, however, the most important damages are caused by insects that attack the chestnut fruit as *Cydia splendana* (Lepidoptera) and *Balaninus (Curculio) elephas* (Coleoptera) (CABRAL and NEVES, 1987). *Cydia splendana* has been linked in recent years with major losses in nut production for the Madeira Island (up to 40% losses in some localities) and Terceira Island (30-40% for 2005 and 2006, respectively), but also in Trás-os-Montes e Alto Douro mainland province (BENTO *et al.* 2007, FARIA *et al.* 2007, LOPES *et al.* in press).

Studies regarding the nematofauna associated with chestnut have been done by MACARA (1987), reporting nine species of phytoparasitic nematodes. Correlations between nematofauna and tree decline wowever not found.

REVIEW OF *CRYPHONECTRIA PARASITICA* (MURRIL) BARR

NOTES ON *C. PARASITICA* TAXONOMY AND MORPHOLOGY

Cryphonectria parasitica (Murr.) Barr. [Syn. *Endothia parasitica* (Murril) P. J. Anderson & H. W. Anderson] (anamorph: *Endothiella*) is the causal agent of chestnut blight disease which infects tree species in the *Castanea* and *Quercus* genera. Taxonomically, *C. parasitica* is included by KIRK *et al.* (2001) in **Ascomycota** phylum, **Ascomycetes** class, **Sordariomycetidae** subclass, **Diaporthales** order, Valsaceae family and *Cryphonectria* genus. Recently a new family **Cryphonectriaceae** was proposed in the Diaporthales by Gryzenhout *et al.* (2006a) including *Cryphonectria* genus.

The chestnut blight fungus was first described in 1906 as *Diaporthe parasitica* Murr. and renamed in 1912 as *Endothia parasitica* (Murril) P. J. Anderson & H. W. Anderson (GRIFFIN and ELKINS, 1986).

Cryphonectria was considered as a synonym of *Endothia* (SHEAR *et al.*, 1916; BARR 1978; ROANE 1986) until the work of Barr in 1978. The taxonomy of the genus *Endothia* was strongly modified by BARR (1978) in his Diaporthales monograph; *Cryphonectria* was separated from *Endothia,* based on the configuration and texture of the stromata, and the septation and shape of the ascospores (GRIFFIN and ELKINS 1986; ROANE et al 1986). The reason for this separation lies in the morphological discrepancies of both species. *Cryphonectria* species are morphologically characterized by semi-immersed stroma with weak development in the bark. Stromatic tissue is predominantly prosenchymatous and perithecia are often forced into a valsoid configuration by surrounding bark tissue (BARR, 1978; MICALES and STIPES, 1987). In opposition, the stromata of *Endothia* spp. is morphologically characterized by a strong development in the bark, with predominant pseudoparanchymatous tissue and perithecia usually born in an upright, diatrypoid configuration (BARR, 1978; MICALES and STIPES, 1987). In addition, the ascospores of *Cryphonectria* spp. range from fusoid to ellipsoid in shape and are one-septate, while *Endothia* ascospores present a cylindrical to allantoid shape and are aseptate. As a result of these observations, *Cryphonectria* was placed in the Valsaceae family and *Endothia* in the Gnomoniaceae family. Only three of the 13 species originally included in *Endothia* were retained in this genus (*E. gyrosa*, *E.*

singularis and *E. viridistroma*), the remaining species being transferred to the *Cryphonectria* genus.

Recent phylogenetic studies on the members of the **Diaporthales**, [morphological characteristics include perithecia with long necks that are located in pseudostromata with no paraphyses and thick-walled asci that are either evanescent with short stalks or intact (KIRK *et al.*, 2001)] have shown that the order includes a number of distinct phylogenetic groups. These groups represent families of **Gnomoniaceae** G. Winter, **Melanconidaceae** G. Winter, **Valsaceae** Tul. & C. Tul, **Diaporthaceae** Höhn ex Wehm., and **Togniniaceae** Réblová, L. Mostert, W. Gams & Crous (CASTLEBURY *et al.,* 2002; GRYZENHOUT *et al.,* 2006a). This classification was possible due to DNA sequence comparisons performed by CASTLEBURY *et al.* (2002). New groups representing undescribed families also have emerged and they have been referred to as the *Schizoparme*, *Cryphonectria-Endothia* and *Harknessia* complexes (CASTLEBURY *et al.*, 2002).

The new family **Cryphonectriaceae** (GRYZENHOUT *et al.*, 2006a,b) was defined to accommodate genera from the *Cryphonectria-Endothia* complex. These genera could be distinguished from those in other families or undescribed groups of the Diaporthales by the formation of orange stromatic tissue at some stage of their life cycle, a purple reaction in KOH and a yellow reaction in lactic acid associated with pigments in the stromatic tissue or in culture, with phylogenetic support from analysis of ribosomal and β-tubulin genes (CASTLEBURY *et al.*, 2002).

The new family Cryphonectriaceae includes nine genera: ***Cryphonectria*** (Sacc.) Sacc. & D. Sacc. (*Cryphonectria sensu stricto*)*,* ***Chrysoporthe*** Gryzenhout & M.J. Wingf.*,* ***Amphilogia*** Gryzenh. & M.J. Wingf.*,* ***Rostraureum*** Gryzenh. & M.J. Wingf.*,* ***Microthia*** Gryzenh. & M.J. Wingf, ***Holocryphia*** Gryzenh. & M.J. Wingf., [all species in these five genera belonged to old *Cryphonectria* genus – "*Cryphonectria senso* Barr" (BARR, 1978) (see Table 6)], ***Endothia*** (Schw.) Fr., and ***Ursicollum*** Gryzenh. & M.J. Wingf genera, as well as the provisory ***Aurapex*** Gryzenhout & M.J. Wingf. genus (GRYZENHOUT *et al.*, 2005a,b; 2006a,b,c) (see Table 6). To facilitate the distinction between different diaporthalean genera with orange stromatic tissue, GRYZENHOUT *et al.* (2006c) provided a key that can be consulted in Annex IV.

Recent taxonomic revisions have restricted the name *Cryphonectria* (*sensu stricto*) only to four species, ***C. parasitica*** (Murr.) Barr., ***C. radicalis*** (Schwein.: Fr.) M.

E. Barr, ***C. macrospora*** (Tak. Kobay. & Kaz. Ito) M.E. Barr and ***C. nitschkei*** (G.H. Otth) M.E. Barr: (GRYZENHOUT *et al.*, 2006a,b,c).

C. parasitica is the type species of the genus *Cryphonectria* (*sensu stricto*) and the only one, inside this genus, considered as a primary plant pathogen.

Concerning the species retained by Barr in the *Endothia* genus (BARR, 1978), only *E. viridistroma* stays nowadays out of that genus (MYBURG *et al.* 2004b).

Morphological characterization of *C. parasitica*:

"Stromata scattered, often confluent, at first immersed in the periderm, becoming erumpent, valsoid, yellow to yellowish brown, prosenchymatous, composed of loose weft of hyphae in the upper part and hyphae mixed with substrate cells in the lower region, up to 3 mm wide and 2.5 mm high. Perithecia grouped, more or less oblique, globose to depressed globose, up to 400 µm broad with dark brown to black, cylindrical, ostiolar beak converging through the stromatic disc and exposing the papilla at the surface with the pore lined on the inside by hyaline, filiform, periphyses; beaks up to 900 x 200 µm. The perithecial wall is composed of hyaline to subhyaline, peseudoparenchymatous cells towards the outside and hyaline, elongated, more or less rectangular cells towards the inside. The neck is composed of vertically elongated, dark brown cells. Asci clavate to clavate cylindrical, thin walled, unitunicate, 8 spored, 32—55 x 7—8.5 µm, with a non-amyloid apical apparatus, loosening from the hymenium and freely floating in the centrum cavity. Ascospores irregularly biseriate in the ascus, hyaline, one septate, not or very slightly constricted at the septum, elliptic, usually straight, rounded at the ends, 7—12 x 3—5.5 µm. Conidiomata pseudostromatic, immersed, erumpent, separate or aggregated, yellow to yellowish brown, globuse, pulvinate, unilocular or complex and multilocular or convoluted, variable in size up to 300 µm wide, with one ostiole. The wall is composed of subhyaline to pale brown, pseudoparanchymatous cells and between locules the cells are somewhat elongated. Conidiophores branched, septate, hyaline, smooth, up to 60 µm long, 1.5 µm wide, formed from the cells lining the condiomatal cavity. Conidiogenous cells enteroblastic, phialidic, determinate, integrated, terminal or lateral, usually below a septum, hyaline, tapering at the apex with an indistinct channel and collarette. Conidia hyaline, one celled, ellipsoidal, to somewhat bacilliform, 3—5 x 1—1.5 µm" (SIVANESAN and HOLLIDAY, 1981).

Table 6 – Genera and species included in the new family Cryphonectriaceae (* plant pathogens) (based in BARR, 1978; CASTLEBURY *et al.,* 2002; MYBURG *et al.* 2004a,b and GRYZENHOUT *et al.*,2004; 2005a,b; 2006a,b,c,d).

Cryphonectriaceae new family			Genera	Species
	Before Barr (1978)	*Cryphonectria senso* Barr (after Barr, 1978)	***Cryphonectria*** (Sacc.) Sacc. & D. Sacc. (*sensu stricto*)	****C. parasitica*** (Murr.) Barr. [T] (*Diaporthe parasitica →Endothia parasitica*) ***C. radicalis*** (Schwein.: Fr.) M. E. Barr ***C. macrospora*** (Tak. Kobay. & Kaz. Ito) M.E. Barr ***C. nitschkei*** (G.H. Otth) M.E. Barr
			Chrysoporthe Gryzenhout & M.J. Wingf. .	****Chr. cubensis*** (Bruner) Gryzenh. & M.J. Wingf. ← ←*C. cubensis* (=*D. cubensis*) ****Chr. austroafricana*** Gryzenh. & M.J. Wingf. ****Chrysoporthella hodgesiana*** Gryzenh. & M.J. Wingf (anamorph) ****Chr. doradensis*** Gryzenh. & M.J. Wingf. ****Chr. inopina*** Gryzenh. & M.J. Wingf.
			Amphilogia Gryzenh. & M.J. Wingf.	***A gyrosa*** (Berk. & Broome) Gryzenh. &M. J.Wingf. [T] ***A. major*** Gryzenh. & M. J. Wingf.
			Rostraureum Gryzenh. & M.J. Wingf.	****R. tropicale*** Gryzenh. & M. J. Wingf. ***R.. longirostris*** (Earle) Gryzenh. & M. J. Wingf. ← ← *C. longirostris*
			Microthia Gryzenh. & M.J. Wingf	***M. havanensis*** (Bruner) Gryzenh. & M.J. Wingf. ← ← *C. havanensis* ***M. coccolobae*** *(Vizioli) Gryzenh. & M.J. Wingf.* ← ← *C. coccolobae* (=*C. coccolobii*)
			Holocryphia Gryzenh. & M.J. Wingf.	****H. eucalypti*** (M. Venter & M.J. Wingf.) Gryzenh. & M.J. Wingf. ← ← *C. eucalypti*
			Endothia (Schw.)Fr	****E. gyrosa*** (Schwein.: Fr.) Fr. [T] ***E. singularis*** (Syd. & P. Syd.) Shear & N. E.Stevens
	New genera		***Ursicollum*** Gryzenh. & M.J. Wingf	***U. fallax*** Gryzenh. & M.J. Wingf.
			Aurapex Gryzenhout & M.J. Wingf. (provisory genus)	****A. penicillata*** Gryzenh. & M.J. Wingf. (anamorph)
			Cryptodiaporte corni from *USA* and Unidentified isolates from Indonesia (probably new genera)	

C. PARASITICA SPREAD AND DISTRIBUTION

With a current distribution comprising Africa, Europe, Northern and Southern Asia as well as Southeastern Asia, the USA and Canada, chestnut blight was first observed in North American, in the beginning of the 19th century, when American chestnut (*Castanea dentata* [Marsh.] Borkh) trees started to die in the New York Zoological Garden (ANAGNOSTAKIS, 1987). The disease spread rapidly and caused the almost complete destruction of the American chestnut population within a few decades, an unparalleled fact in forest history (ROANE *et al.* 1986; ANAGNOSTAKIS, 1988). The fungus was apparently introduced into New York City and Long Island on nursery stock of Oriental chestnut, possibly from Japan (the first record of Japanese chestnut trees imported to the USA is from 1876). This hypothesis is supported by the fact that the fungus was found in native Chinese chestnut (*C. molissima*) in 1913, and in 1915 in native Japanese chestnut (*C. crenata*) (SHEAR and STEVENS 1913, 1916; ROANE *et al.* 1986; ANAGNOSTAKIS 2001).

A similar epidemic was observed in European chestnut stands (*Castanea sativa* Mill.) following the first identification of the disease in northern Italy in 1938 (BIRAGHI, 1946). Although this is the main reported date for the majority of authors, previous references reporting the existence of the disease exist: Bruges, Belgium (1924); Cronberg, Germany (1927); and Alcaide, Portugal (1929) (BALDACCI and PICCO, 1947). After the 1930's the disease rapidly spread throughout Italy and most of Southern Europe. Today, with the exception of the coppice stands in the southern UK and the scattered chestnut stands in the Netherlands, all the European chestnut stands have been infected by *C. parasitica* (HEINIGER and RIGLING, 1994; ROBIN and HEINIGER, 2001). Some references claim that cryptic introduction occurred much earlier and passed unnoticed due to the pre-existent wide-spread decline of chestnut stands mainly caused by *Phythophtora* spp., commonly known as ink disease (ROBIN and HEINIGER, 2001; BRAGANÇA *et al.*, 2005). Japanese chestnut, *Castanea crenata*, which is resistant to ink disease, planted during and after the 1920's in Italy (DEL GUERRA, 1948), France, Spain (DARPOUX *et al.*, 1957) and Portugal (FERNANDES, 1949) aiming to fight the wide spread ink disease, might have been unintentionally a vector for cryptic dispersal of chestnut blight.

C. parasitica is a weak pathogen on Asian chestnuts (JONES *et al.*, 1980; ROANE *et al.* 1986) and most probably, just as in the USA, it was imported into Europe together

with Asian chestnut plants (ANAGNOSTAKIS, 1987). This is suggested by the fact that in Spain and Portugal the presence of *C. parasitica* was first noticed on *C. crenata* (BIRAGHI, 1948; CÂMARA, 1929). However, many European chestnut stands recovered from the disease since the occurrence of superficial non-lethal chestnut blight cankers was observed. This recovery has been attributed to hypovirulence, caused by the dsRNA hypovirus CHV1, a phenomenon in which fungal viruses significantly reduce virulence and sporulation of *C. parasitica* (GRENTE, 1965; ANAGNOSTAKIS, 1982; HEINIGER and RIGLING, 1994; ALLEMANN *et al.*,1999).

C. parasitica can occur in numerous and unevenly distributed vegetative compatibility (vc) types. Initially, in a newly-infected area, only one type appears, but later on colonization by more vc types is expected. The spread of the fungus may be delayed by the occurrence of hypovirulence, mainly in older infection spots and mostly with the hypovirulent vc type that initially colonized the trees of that region. It has been assessed that the reduced virulence of hypovirulent isolates retards the spreading of the pathogen. However, any newly-established virulent vc type triggers another phase of aggressive infection and spread. As the rate of hypovirulence transmission increases, mainly within the same virulent vc type, spread of the fungus supposedly slows down and tree mortality declines (MILGROOM and CORTESI, 2004).

Hypovirulent CHV1 isolates have been established for several decades in some European stands (*eg.* France, Italy, Switzerland, Croatia, Greece), where blight severity is significantly lower compared to chestnut localities where blight colonization is recent and where CHV1 has not occurred. In the later situations, blight epidemics may take place and be devastating (HEINIGER and RIGLING, 1994; ROBIN and HEINIGER 2001). Thus, chestnut blight distribution is not directly correlated with high infection rates while lower infection rates, are a direct function of the presence of CHV1 hypovirulence.

At present time, due to the inexistence of continuous chestnut stands in Central Europe, the expansion rate of the disease has decreased (HEINIGER and RIGLING, 1994), however, reports of new affected areas in Europe[e.g. Southwest of Germany (SEEMANN, 2001) and the Portuguese Madeira Island (BRAGANÇA *et al.*, 2004; 2005)] and in Asian continent still occur [e.g. Iran (KAZEMPOUR *et al.* 2006)]. Actually *C. parasitica* is in EPPO list quarantine organism (EPPO A2 list nº 69 Annex II/A2 2006). The actual distribution of *C. parasitica* in the world is shown in Figure 1.

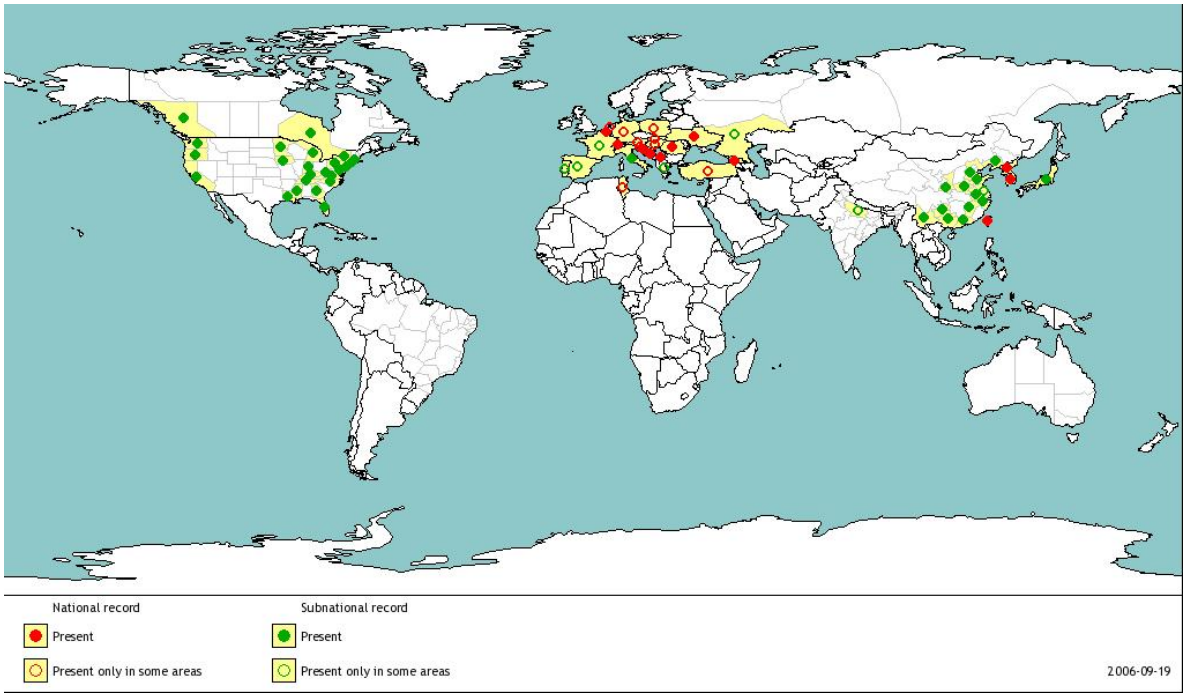

Figure 1 - Map of *Cryphonectria parasitica* distribution in the world.
(Available at www.eppo.org/QUARANTINE/fungi/Cryphonectria_parasitica/ENDOPA_map.htm)

BIOLOGY OF *C. PARASITICA* – SYMPTOMS, SIGNS AND CONTROL OF THE DISEASE

C. parasitica is a wounding parasite that infects branches and stems. The most common symptoms on European and American chestnuts are foliage yellowing, wilting and dieback (caused by the girdling of attacked branches) (Figure 2). Some symptoms are visible from a distance during the growing season of the tree, since dry leaves remain in the twigs and copious epicormic shoots are produced by the plant below the cankers (ROANE et al., 1986; GRIFFIN, 1986; ANAGNOSTAKIS, 1994; HEINIGER and RIGLING, 1994) (Fig 2 C, D). These symptoms are often the first indication of blight infections, since the cankers are not easily seen on mature bark in the initial stages of infection (ANAGNOSTAKIS, 1987).

The signs of the disease comprise the presence of the reproductive structures of the parasite - pycnidia and perithecia – in orange-brown stromata emerging from branches or the main stem (Figure 3). As disease progresses, the smooth bark of young branches becomes reddish, sunken (Figure 3) and the fungus proliferates in the

cambium and in the bark tissue forming pale brown mycelial fans (ROANE *et al.*, 1986; ANAGNOSTAKIS, 1987; HEINIGER and RIGLING 1994). Yellow tendrils of conidia (cirrhi) extrude from the stromata in wet weather (Figure 3).

The physiological response of the plant results in bark cankers. The adjacent or enclosing bark of the canker may be smooth, sloughed, or in some cases, slightly swollen and split (Figure 4). In young trees, cankers commonly occur at the base of the tree or in association with branch stubs. Usually the roots are not infected and stumps will sprout again. However, there are references that describe infected roots below soil level (GRIFFIN, 1986) and in these cases new shoots could be re-infected (GRIFFIN *et al.*, 1991). The mechanisms of *C. parasitica* diffusion are not fully understood, mainly because it is not known (with seldom exceptions) the processes conducing to the dissemination phase in the life-cycle of the fungus at different environmental conditions (ROBIN and HEINIGER, 2001).

C. parasitica is heterothallic with a low outcrossing rate, showing a clonal or self-fertile life cycle. The reproductive strategy of the fungus determines its spread. *Cryphonectria* spp. also present a broad number of vc types and disease severity rates ranging from low to high virulence depending on the presence or absence of CHV1 viral infections (MILGROOM, 1995). In the USA, where vc type diversity is higher than in Europe, most of the ascospores are discharged in autumn and are the main source of primary inoculum responsible for the establishing of new cankers (ANAGNOSTAKIS, *et al.*, 1998). As for Europe, data have shown that ascospores are discharged from spring until autumn, with a peak of spore release in spring, after rainfall, when chestnut is more susceptible to blight (GUÉRIN *et al.*, 1998; ROBIN and HEINIGER, 2001).

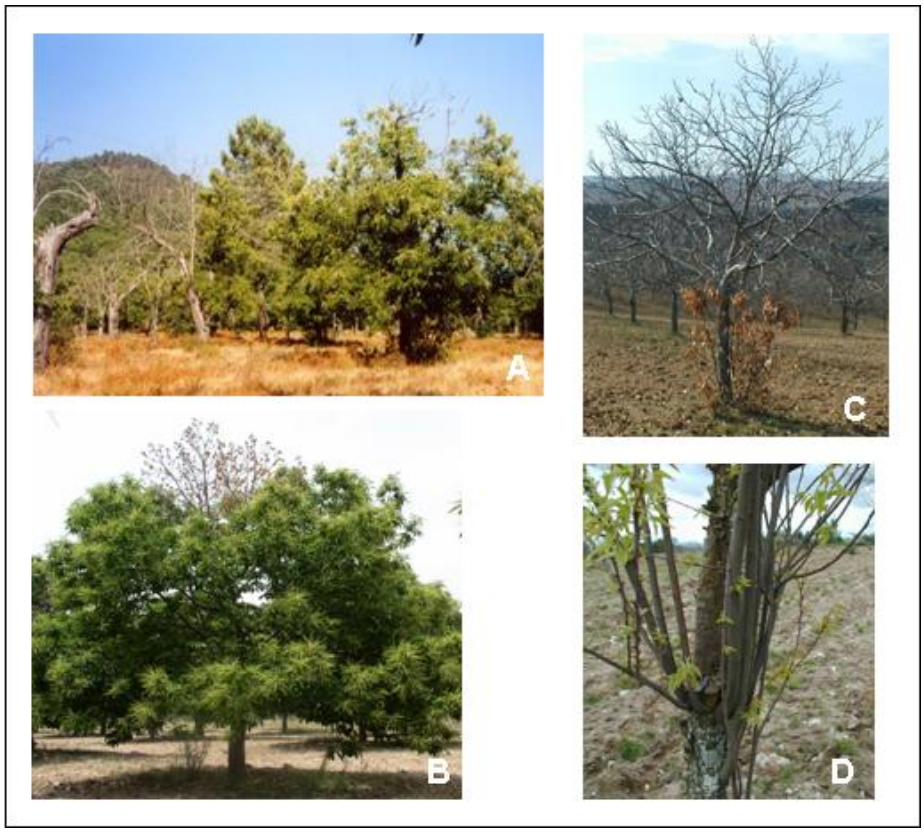

Figure 2 – Disease symptoms of chestnut blight caused by *Cryphonectria parasitica* on *Castanea sativa*: A - dead trees killed by chestnut blight. B - die-back of branches with dead leaves still attached. C and D- epicormic shoots arising below cankers.

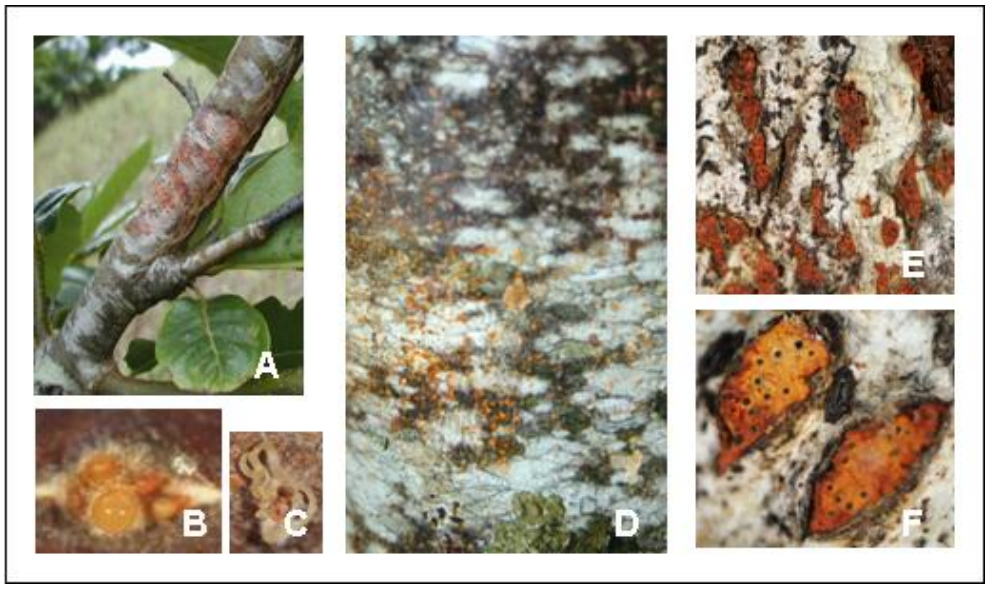

Figure 3 – *Cryphonectria parasitica* reproductive structures on *Castanea sativa*: A - sunken canker with fruiting structures, B - orange-yellow pycnidiospores exuding from pycnidia , C - tendrils of pycnidiospores on tree bark. D - orange-yellow pychnidial stromata , E– perithecial stromata emerging through bark, F - tranversal cut showing stromata and perithecia necks (dark dots).

Figure 4 – *Castanea. sativa* cracked bark due to *Cryphonectria parasitica* infection.

In order for the inoculum to penetrate the epidermal layer of chestnut, mechanical or natural lesions must be present. However, spores can remain latent in the bark until suitable conditions for penetration and development arise (GRIFFIN, 1986; ROANE *et al.*,1986; GUERIN and ROBIN, 2003). When conditions for germination are favorable, conidia or ascospores from virulent *C. parasitica* develop a germ tube and hyphae ramify in fresh lesions of the bark. Invasion is accomplished through enzymes which can break the plant's natural defenses. *C. parasitica* produces several polysaccharide-degrading enzymes that lead to the maceration of the cell wall. These enzymes are more active at a pH of approx. 5.5 - the pH of the inner bark of chestnut. The resulting hyphae form mycelial fans that grow progressively into the inner bark/cambium region producing oxalic acid in the disease front, which alters the pH to approx. 2.8. The host cells of the tree are killed by the chemical and mechanical action of the pathogen leading to the disruption in the xylem flow. The initial lesion induces the tree to produce a periderm barrier which, when completely formed, limits the mycelium spread into adjacent bark tissues (MCCARROL and THOR, 1985; HEINIGER and RIGLING, 1994).

In both *C. sativa* and *C. dentata* the infection and colonization process is very fast and most of the times the mycelium reached the periderm layer before its completion allowing the fungus to invade all the cambial tissue (ANAGNOSTAKIS and HILLMAN, 1992). The fast rate of development and growth of mycelial fans and the high acidity at the canker margin appear to be the two most important factors in the pathogenesis of *C. parasitica* (GRIFFIN and ELKINS, 1986). The rapid growth of mycelial fans requires a large nutrient source and some authors have suggested that *C. parasitica* uses chestnut tree tannins as the main nutritional source for canker expansion (GRIFFIN and ELKINS, 1986).

The abiotic condition that the chestnuts are subjected to, namely seasonal variations in temperature and moisture and water drought stress, as well as the initial infection point in the tree, are critical external factors which can influence the pattern of development of the blight and its progression through the host (RUSSIN and SHAIN, 1984; GAO and SHAIN, 1995; GUÉRIN and ROBIN, 2003).

Long distance transportation of ascospores and mycelial particles can be achieved by animal vectors such as birds and insects and also by wind. Conidia are disseminated by insects, birds, snails, mammals and by rain (GRIFFIN, 1986; ANAGNOSTAKIS, 1987; HEINIGER and RIGLING, 1994). Man is also an important vector

in the spread of the disease when infected chestnut plants, wood or nut shells are reallocated for commercial purposes (JAYNES and DEPALMA, 1994). Cultural practices can also contribute to the spread, when infected material is used for grafting or when pruning is not performed aseptically. Branches colonized by the fungus can be asymptomatic without signs of the disease (BISSEGGER and SIEBER, 1994; GUÉRIN and ROBIN, 2003). In order to reduce the risk of infection by chestnut blight, pruning and thinning should be carried out only during the period of lowest receptivity and susceptibility to the fungus, *i.e.* when the availability of ascospores is minimal. These conditions occur in winter (GUÉRIN and ROBIN, 2003).

The control of chestnut blight, although a difficult endeavor, imperatively has to include an approach comprising the implementation of appropriate silvicultural measures (such as chirurgical pruning and canker removal). In addition, biological control program aiming the use of hypovirulent isolatess should also be implemented. Ecological sanitary measures may reduce the amount of available inoculum albeit alone will have a relatively small impact on the progression of the disease. Fungicides may occasionally be applied in seldom situations, i.e., graft protection (HEINIGER and RIGLING, 1994; MILGROOM and CORTESI, 2004).

Biological control has shown to be effective in some cases. Nevertheless, the success of biocontrol depends of achieving the integration of many factors (MILGROOM and CORTESI, 2004). If in Europe successful cases have been reported with the introduction of biocontrol measures, these do not show a parallel with the situation in North America. In this country the success of biological control is much lower. These occurrences may be correlated with the fact that the American chestnut species, *C. dendata*, is much more susceptible to the fungal pathogen. Moreover, there is a higher difficulty in the dissemination of hypovirulent isolatess due to the high diversity in vc groups of *C. parasitica* in this country. The differences in the success of biological control achieved in both regions can be also linked with the differences in the dsRNA hypovirulent isolates present in both continents. Biological control with hypovirulence is especially attractive for managing chestnut blight in forest chestnut stands since ecological sanitation or fungicide treatments, are economic and environmental impracticable (HEINIGER and RIGLING, 1994).

THESIS OUTLINE

After a general introduction regarding chestnut tree and *C. parasitica* (PART I), a description of the Portuguese situation concerning the distribution of chestnut and the dispersal of chestnut blight disease will be presented (PART II - Chapter II-1). To understand the distribution of the disease and determine the dispersal factors of *C. parasitica* in Portugal, a study case is established in the most important Portuguese region of chestnut production (PART II - Chapter II-2). The population genetic variability of *C. parasitica* is investigated using PCR based fingerprinting methods, mating types (*MAT* genes) and vegetative compatibility types. Screening for naturally occurring hypoviruses and *a posteriori* induced hypovirulence were conducted to promote future biological control measures in the country (PART III - Chapter III-1). Sequencing of the nuclear ITS region of rDNA and the genes encoding for β tubulin 1 and β tubulin 2 was performed in order to discriminate the phylogeny of *Cryphonectria* species found in Portugal during the monitoring phase of the project (PART III - Chapter III-2). Finally, the main findings and outcomes of this thesis are present.

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PART II

CHESTNUT BLIGHT DISEASE IN PORTUGAL

CHAPTER II-1

SURVEY AND GEOGRAPHIC DISTRIBUTION OF CHESTNUT BLIGHT IN PORTUGAL

SURVEY AND GEOGRAPHIC DISTRIBUTION OF CHESTNUT BLIGHT IN PORTUGAL

PROSPECÇÃO E DISTRIBUIÇÃO GEOGRÁFICA DO CANCRO DO CASTANHEIRO EM PORTUGAL

HELENA BRAGANÇA[1], SOFIA SIMÕES[1], MIGUEL CAPELO[1], JOSÉ MARCELINO[2], NATÉRCIA SANTOS[1]

ABSTRACT

Cryphonectria parasitica, the fungal pathogen causing chestnut blight disease, was first described in Portugal (1929) on bark samples from the exotic Japanese chestnut, *Castanea crenata*, collected from young plants in Beira Interior Province. After this early report, only in 1989 two disease foci were found on the native European chestnut, *C. sativa*, in Trás-os-Montes e Alto Douro Province. To assess the current distribution of chestnut blight in Portugal (mainland, Azores and Madeira archipelagoes) a nationwide monitoring program was implemented. A major cooperative effort, involving all the Portuguese Forest and Agricultural Services, was created for this endeavor and coordinated by the National Forestry Station (Estação Florestal Nacional). A total of 191 chestnut stands was surveyed across the entire country. *C. parasitica* was isolated from 56.5% of the sampling sites (108 sites). Sampled parcels were digitally geo-referenced and mapped. In addition, a collection of *C. parasitica* isolates has been established and the study of the molecular variability of *C. parasitica* in Portugal is underway.

Key-words: *Cryphonectria parasitica, Castanea sativa,* Portugal

RESUMO

Cryphonectria parasitica, o fungo fitopatogénico responsável pelo cancro do castanheiro, foi descrito pela primeira vez em Portugal (1929) em amostras da casca de castanheiro Japonês, *Castanea crenata*, recolhidas em plantas jovens na Beira Interior. Depois detecção inicial, apenas em 1989, dois focos da doença foram encontrados em castanheiro Europeu, *C. sativa*, na província de Trás-os-Montes e Alto Douro. Para determinar a distribuição actual do cancro do castanheiro em Portugal (continente e Regiões Autónomas da Madeira e Açores), foi implementado um programa nacional de monitorização da doença. Para o efeito houve intensa cooperação entre os Serviços Florestais e Agrícolas do país, com coordenação da Estação Florestal Nacional. Foram visitadas, em todo o país, 191 parcelas de castanheiro. *C. parasitica* foi isolado em 56,5% da amostragem (108 parcelas). As parcelas amostradas foram digitalmente georeferenciadas e cartografadas. Uma colecção de isolados de *C. parasitica* foi estabelecida encontrando-se em curso o estudo da variabilidade genética da doença em Portugal.

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INTRODUCTION

In Portugal, *Cryphonectria parasitica* (Murrill) Barr was first described by Câmara (1929), on bark samples collected on branches from young plants of *Castanea crenata* Sieb. & Zucc, in the Beira Interior Province, near the village of Alcaide, Fundão. Forest Services Publications reported in 1949 that "no focus of chestnut blight was found in Portugal until now, although we are not certain that it does not exist since many Japanese chestnuts have been imported in the last years" (Fernandes, 1949). The initial infection was probably successfully eradicated, since only in 1989 two disease foci were found on European chestnut (*Castanea sativa* Mill.) in the Trás-os-Montes e Alto Douro Province, the most important Portuguese chestnut-growing region in the Northeast of the country, in Carrazedo de Montenegro (Serra da Padrela -Valpaços) and Parada village (Terra Fria - Bragança) (Abreu, 1992).

After the 4th meeting of the International Commission for Chestnut, in 1958, Yugoslavia, Portugal is included within the range of the fungus and in the geographic distribution of the disease in the Commonwealth Mycological Institute (CMI) records (Caetano, 1990). In 1990 the disease was of mandatory report by governmental ordinance ("Portaria 847/90, 18 Setembro"). In spite of this fact and in opposition to the general rule giving priority to eradicate critical quarantine diseases, no financial support was allocated for this purpose. This measure was only taken in 1998 ("Despacho Conjunto 117/98, 18 Fevereiro") in compliance with the establishment of an eradication program which included logging and incineration of diseased branches or the entire tree (Anastácio, 2001).

During the three years of the program, a total of 82 000 chestnut trees were monitored in Northern Portugal by the Direcção Regional de Trás-os-Montes e Alto Douro services (DRATM). The overall rate of infection at that time was approximately 10%, although major differences among localities were observed. However, the disease was present in all counties. In the Chestnut Protected Denomination of Origin zone (PDO) of "Castanha da Padrela", one of the most important in the country, 61 528 trees were monitored with an infection rate of 100% reported in some localities (Anastácio, 2001).

In subsequent years, the disease spread by natural pathways and/or by the influence of man, intensifying chestnut monoculture due to increasing economical interests and investments in chestnut orchards and forest stands (Abreu & Martins, 2002). Overall, the balance of the program was that the eradication objectives were not totally achieved, although the implementation of eradication measures allowed the decrease of the levels of infection. In addition, the improvement of good cultural practices by the growers, also implemented during the program, was an important factor preventing the fast spread of the disease.

The Portuguese archipelago of Azores ("Região Autónoma dos Açores") reported chestnut blight for the first time in 1993. Two internal reports from S. Miguel and Terceira Islands made by the Estação de Fruticultura Vieira Natividade in Portugal and Institute Nationale de Recherche Agronomique (INRA) in France, described the occurrence of the disease. In addition, Ormonde (1994) describes that symptomatic trees in the Terceira Island were not significantly affected by the disease, thus, hypothetically the hypovirulence could also exist at that time and later Abreu and Martins (2002) referred the existence of an hypovirulent culture from Azores. That situation was also observed *a posteriori* and referred by Bragança et al (2004). In Madeira Island ("Região Autónoma da Madeira"), chestnut blight was detected for the first time in 2002 by the Direcção Regional de Agricultura da Madeira authorities

(DRAM), in chestnut stands of "Comissão de Levadas do Curral e Castelejo", Curral das Freiras village. Laboratorial confirmation of the disease was performed in the Laboratório Agrícola da Madeira (Maia, 2003).

To assess the distribution of chestnut blight and the variability of *C. parasitica* in Portugal, it was crucial to put into action a nationwide monitoring program comprising all chestnut growing regions in Portugal. The experimental design of this work would have to assure both the accurate estimate of the distribution of chestnut blight, which previously was presumed to be centralized only in the Northeast of the country, and the characterization of all the existent populations of the fungus. The study of the genetic diversity in populations of *C. parasitica* and the research for hypovirulent isolates was required to implement, in the nearest future, effective actions preventing further spread of the disease.

In the aim of this work all chestnut growing regions in Portugal were visited and a national collection of *C. parasitica* isolates has been established. Considering the importance of the chestnut area and the incidence of chestnut blight in Trás-os-Montes e Alto Douro, some areas in this province were most intensively prospected. In the course of the survey it was possible to observe signs and symptoms not only of *C. parasitica* but of other chestnut diseases as well. Although not a primary goal of this study, additional information on other fungi found in chestnut stands is also briefly presented.

MATERIAL AND METHODS

- **Experimental design**

The selection of the sites to be monitored for the presence of chestnut blight sought to cover all the area of chestnut in Portugal with the inclusion of the possible highest number of sites previously known to present the disease.

Sample sites were selected considering the information given by the Agriculture and Forest Services and the map of chestnut distribution area in the country. All provinces in continental Portugal, Madeira Island, and three Islands of the Azores were surveyed for chestnut blight in the course of the monitoring program.

In each of the regions, assessment of all chestnut sites (by district parish – "freguesia") with an area >0.5 ha was done. Subsequently, these areas were monitored for the presence of *C. parasitica*. In each district parish, as a rule, sites with previously reported presence of chestnut blight were always monitored. For the remaining selected areas, monitoring sites were randomly selected and then inspected for the presence of symptoms and/or signs of the blight.

Cartographic localization of sampling areas was performed using military cartography and orthophotomaps (paper based and, when available, digital materials). Sampling sites and localities were digitally geo-referenced and mapped using GIS analysis tools through ArcView® 3.2 software (Environmental Systems Research Institute, Inc.). The number of sites monitored per region, as well as the number of *C. parasitica* isolates per site, varied largely according to the distribution of chestnut in Portugal and the incidence of chestnut blight, respectively. Distinct data from the sites (*e.g.* signs and symptoms of disease, stand characterization, environmental conditions) and individual tree descriptions were gathered in order to find correlations with the disease.

Field procedures involved visual assessment of all the trees inside the sites in order to detect chestnut blight symptoms: branch or tree mortality with shrivelled, dried leaves, chestnut blight cankers with longitudinal bark fissures and presence of fungal

stromata or mycelial fans. Based on Robin et al. (2000) each sampling site was approx. 1 ha in size and one canker per tree was sampled for a maximum of ten different trees per site (sporadically, in larger sites more than ten trees were sampled).

- **Isolation and identification**

Using a knife, bark samples (approx. 2 x 2 cm) were removed from the cankers and brought to the laboratory. Small pieces (approx. 2 x 2 mm) were cut out from the bark samples and surface-disinfected by dipping for 15s in 70% ethanol. Then, the samples were rinsed in sterile water, blotted on filter paper and placed on potato dextrose agar (PDA, Difco, Sparks, MD, USA). The plates were incubated at 25ºC in the dark and C. parasitica cultures were transferred to new PDA plates. One isolate per canker was used for further analysis**.**

- **Long-term storage of *C. parasitica* isolates**

Before stocking, the isolates were grown on PDA medium placed in Petri dishes and McCartney bottles, then stored at 25ºC in 24h dark for seven days. Subsequently both containers were transferred to a laboratory bench with natural light during 10 to 14 days to allow profuse production of conidia. *C. parasitica* isolates were stored at 6ºC on McCartney bottles (three bottles per isolate). In parallel, the isolates were also frozen in glycerol and stored at -80ºC as follows: 3-4 pieces of PDA (5x5mm) were cut from sporulating cultures and immersed in 800µL of a 22% sterile glycerol solution (Riedel-deHaën, Seelze-Hannover, Germany) allocated in Cryo-vials tubes (Tenak™), mixed heavily by vortexing and finally frozen in liquid nitrogen before storage.

- **Detection of other phytopathogens**

Chestnut sample tissue with signs of other diseases was observed and if necessary processed in laboratory through slide preparations for microscopic observation and posterior confirmation of the phytopathogenic agent of genus level. In some cases, soil analyses were performed in the forest pathology laboratory to confirm the presence of *Phytophthora* sp. as described by Campbell (1949).

RESULTS

A total of 191 chestnut stands were monitored across the entire country comprising 55 district parishes in 34 counties from all Regional Agricultural Department areas in Portugal mainland, and 25 district parishes from 13 municipality areas in the Archipelagoes). (Figure 1, Table 1)**.** The results obtained herein, and presented in Table 1 and Figures 1 to 6 indicate that chestnut blight has spread into most of the chestnut growing area of Portugal. Signs and symptoms of the disease have been detected in six Regional Agricultural Department areas of the mainland and *C. parasitica* was isolated from four of these six regions. The disease was also found in the Azores and Madeira archipelagoes. A total of 617 *C. parasitica* isolates were obtained from 108 sampling sites (mainly orchards) distributed across four mainland provinces (Minho, Trás-Montes, Beira Interior, Alentejo), the Azores Islands (Pico, Terceira, S. Miguel), and the Madeira Island.

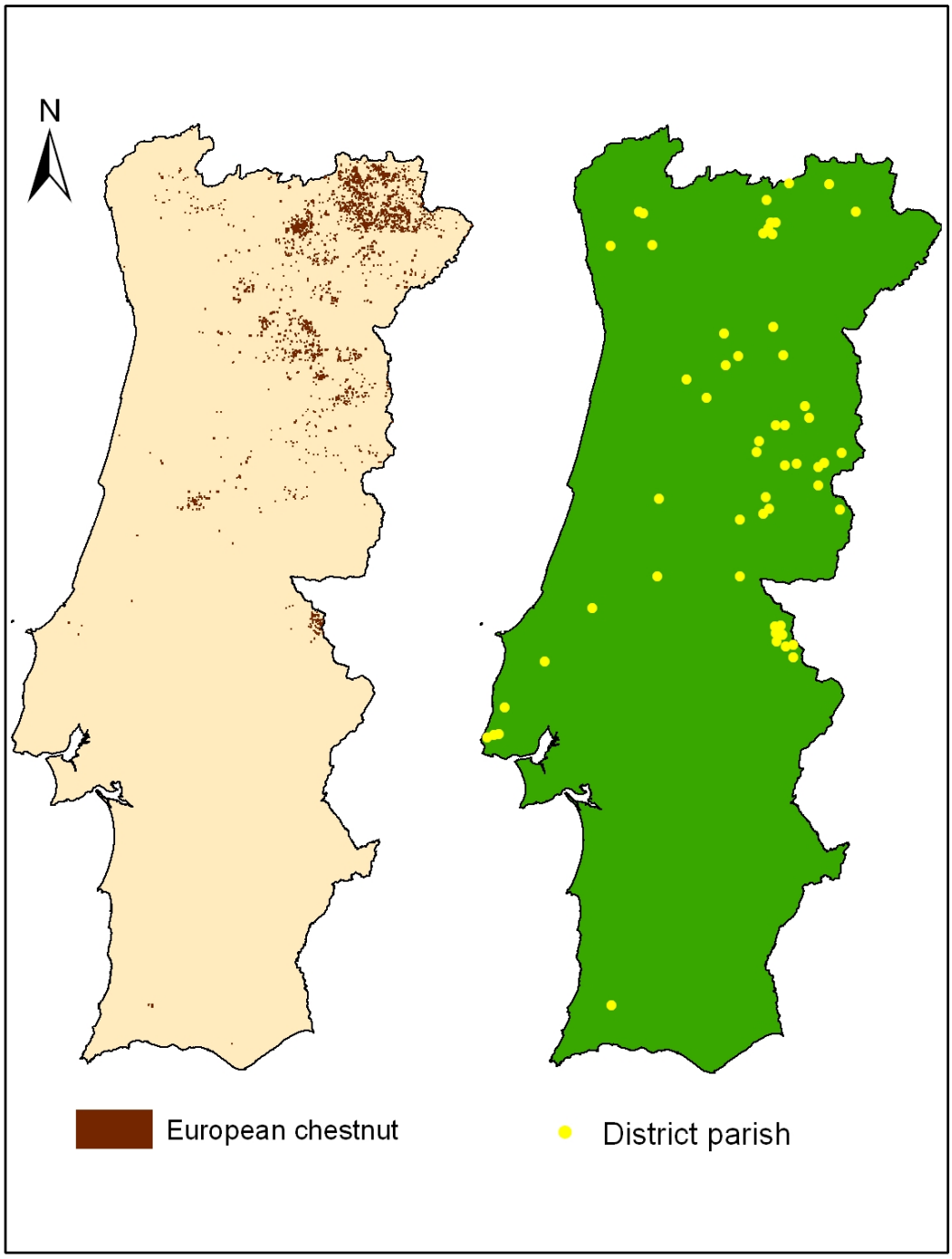

Figure 1 – Portugal mainland: distribution of European chestnut tree and chestnut blight survey by district parish.

Table 1 – Survey and distribution of chestnut blight disease in Portugal

	Regional Agriculture Department in mainland/ Islands	Counties "concelhos"	No. of Sites inspected	No. of Sites with *C. parasitica* isolates
Portugal (mainland)	**DRAEDM**	Vila Verde Amares Barcelos Guimarães	**4 (forest stands)**	**4**
	DRATM	Chaves Valpaços Vinhais Bragança Tarouca Penedono	**73**	**58**
	DRABI	Trancoso Guarda Manteigas Belmonte Sabugal Fundão Penamacor Castelo- Branco Idanha-a-Nova Vila Velha de Rodão	**23 (14 forest stands)**	**6**
	DRABL	Castanheira de Pêra Lousã Vila Nova de Paiva S. Pedro do Sul Viseu	**6 (2 forest stands)**	**0**
	DRARO	Alcanena Ferreira do Zêzere Cadaval Mafra Sintra*	**10 (8 forest stands)**	**0**
	DRAAL	Portalegre Castelo de Vide Marvão	**25 (2 forest stands)**	**7**
	DRAALG	Monchique*	**2**	**0**
Azores ("Região Autónoma dos Açores")	**PICO**	Madalena S. Roque do Pico	**6**	**2**
	TERCEIRA	Angra do Heroísmo Vila Praia da Vitória	**14**	**12**
	S. MIGUEL	Ponta Delgada Ribeira Grande Lagoa Vila Franca do Campo Nordeste Povoação	**18**	**16**
Madeira ("Região Autónoma da Madeira")	**MADEIRA**	Ribeira Brava Câmara de Lobos Funchal	**11**	**3**

*** Includes sites with signs and symptoms of chestnut blight**

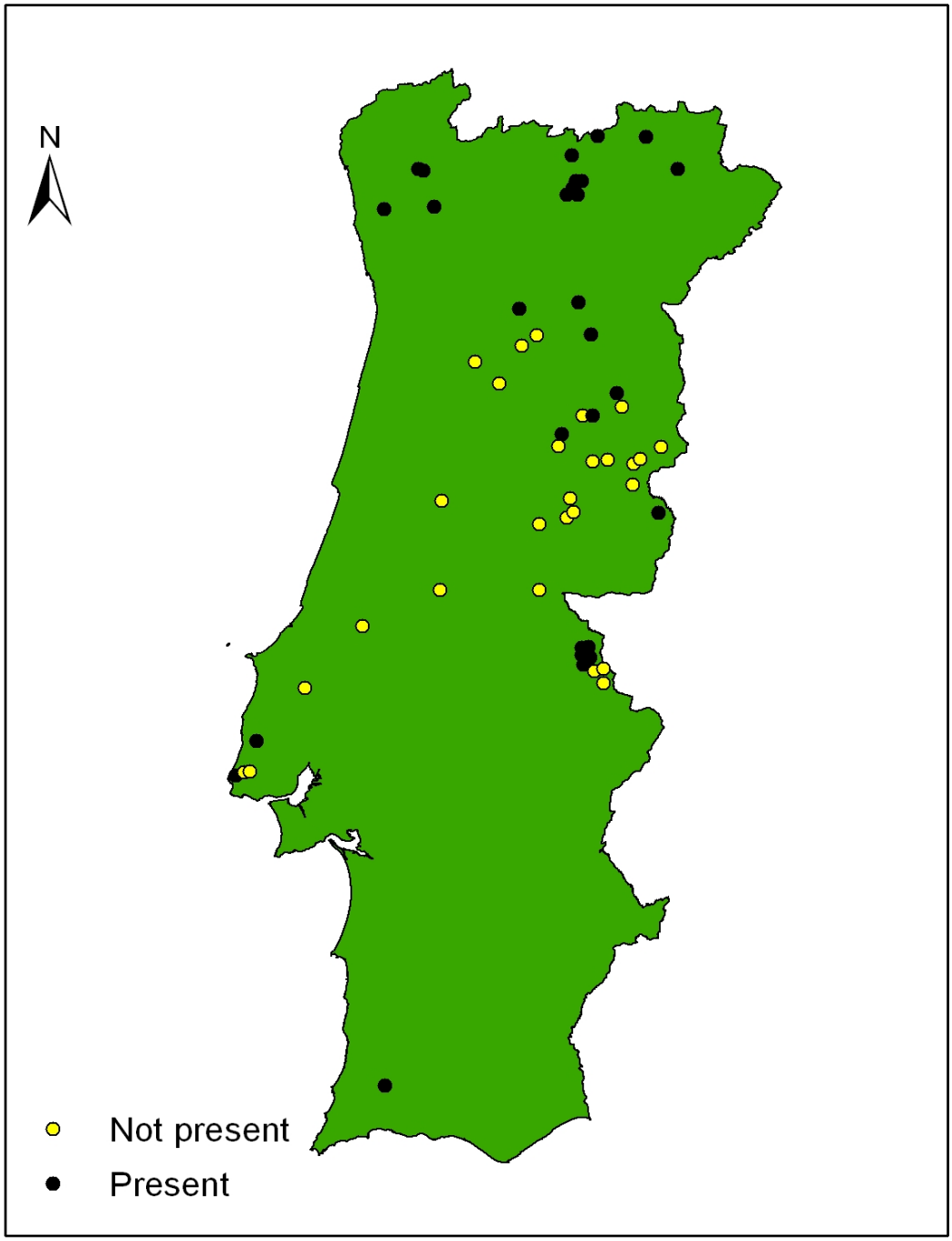

Figure 2 – Geographic distribution of chestnut blight in Portugal mainland (by district parish).

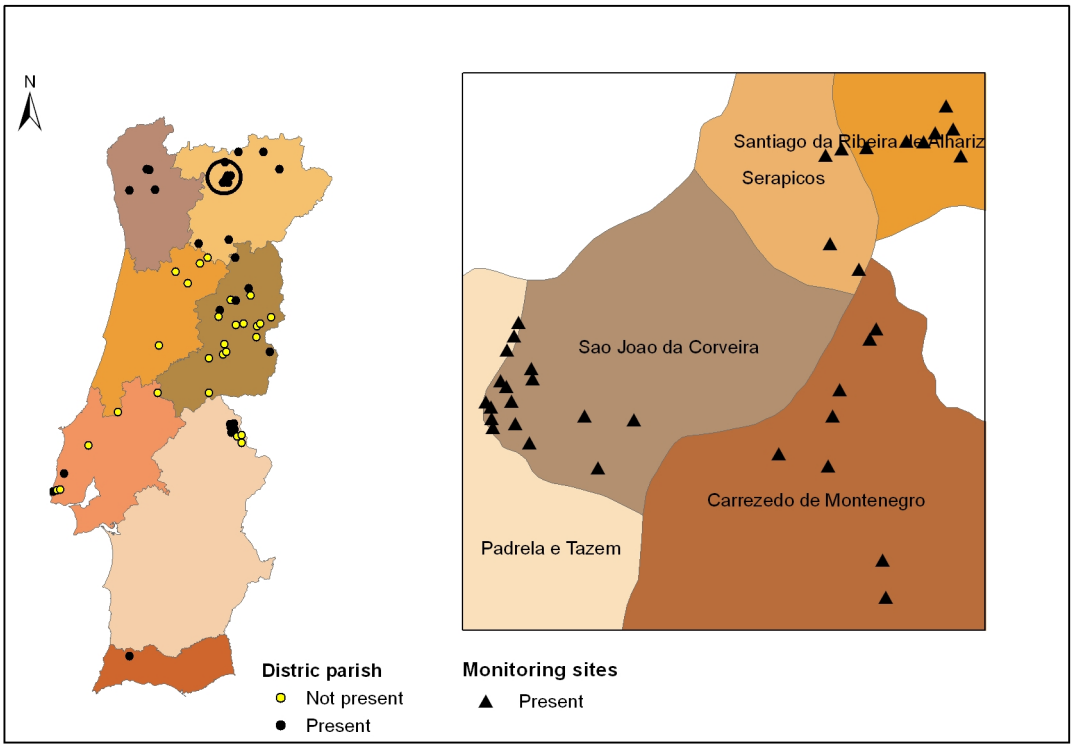

Figure 3 - Geographic distribution of chestnut blight - Valpaços detail (in Trás-os-Montes e Alto Douro).

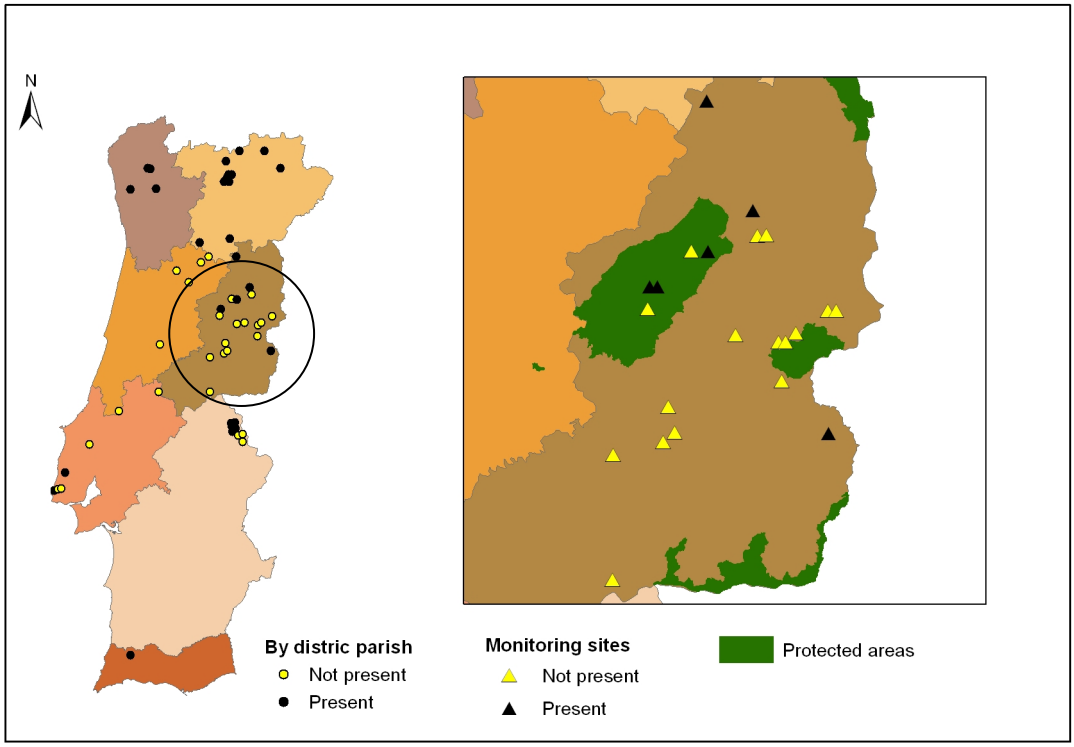

Figure 4 - Geographic distribution of chestnut blight - Beira Interior detail.

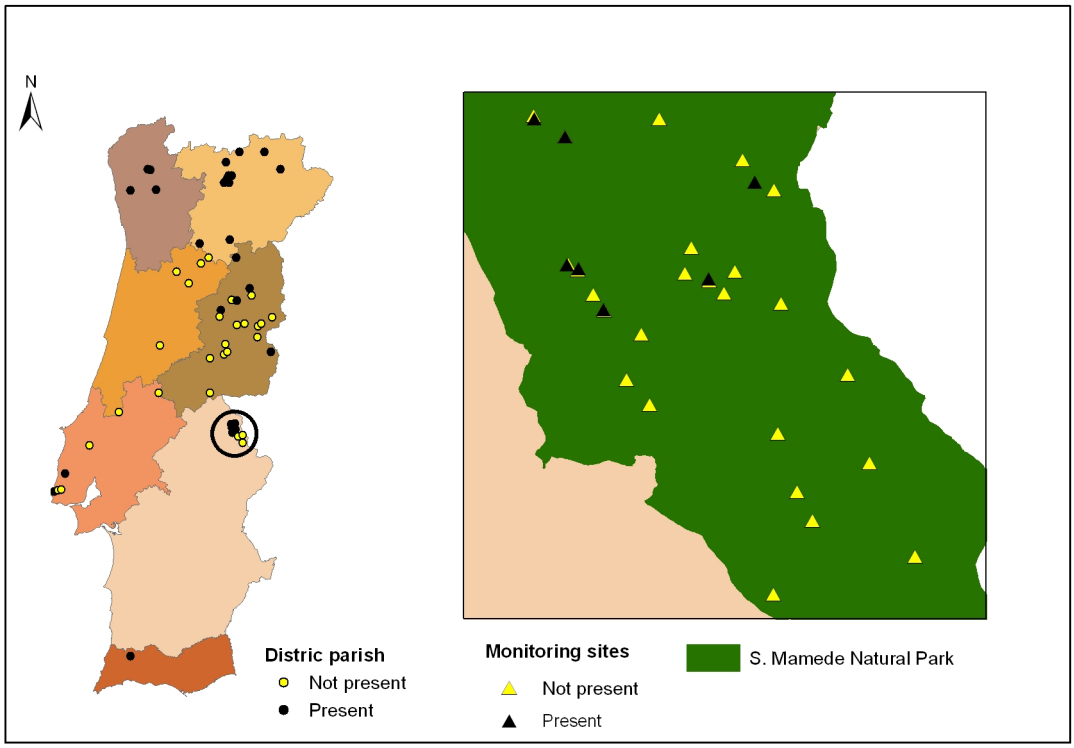

Figure 5 - Geographic distribution of chestnut blight - Alentejo detail.

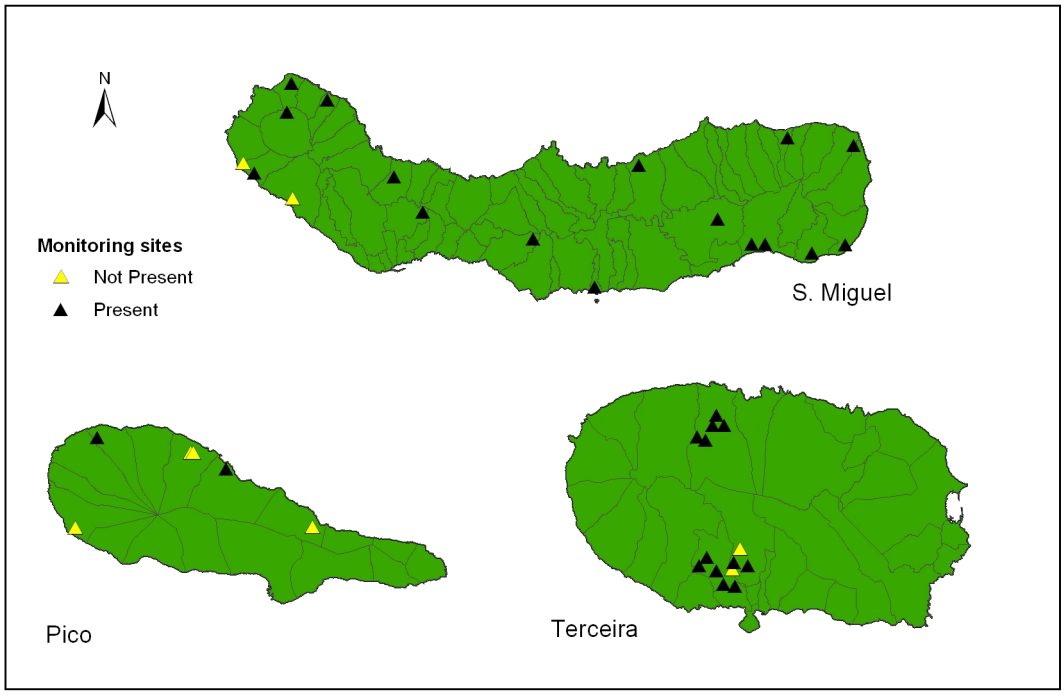

Figure 6 - Geographic distribution of chestnut blight – Azores Islands detail (lines inside the maps represent District parish borders).

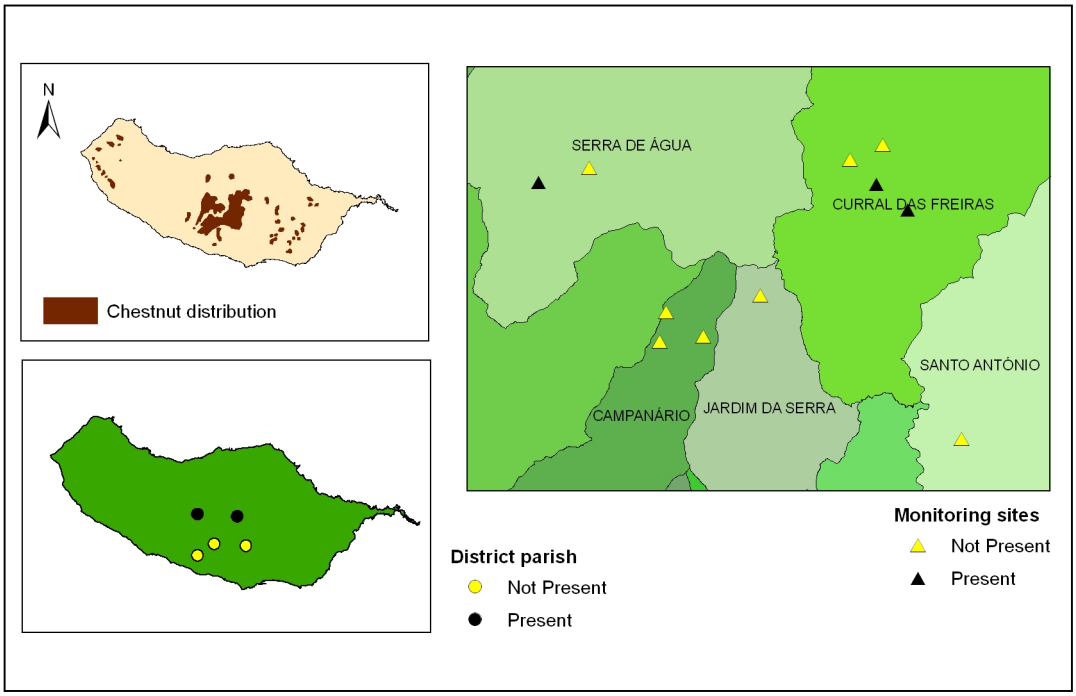

Figure 6 - Geographic distribution of chestnut blight – Madeira Island detail.

Regarding the survey of the other diseases, the following phytopathogenic fungi were identified: *Coryneum* sp., *Cytospora* sp. and *Phytophthora* sp.. Symptoms of ink disease were observed in all visited regions, diffuse cankers caused by *Coryneum* sp. were observed mainly in Trás-os-Montes e Alto Douro region and *Cytospora* sp. was found affecting young plants in São Mamede Natural Park in Alentejo region.

DISCUSSION AND CONCLUSIONS

Portuguese chestnut stands monitored during this work varied in structure and composition. In Trás-os-Montes e Alto Douro, the most important chestnut area in Portugal, most chestnut areas form a continuous surface of orchard stands. In Beira Interior, the type of chestnut stand is heterogeneous, with both small and extended orchards, high forest and coppice areas. Overall, the high forest and coppice stands where the disease was observed were known to have suffered past human interventions, although at a much smaller rate than the orchards. Conversely, the vast majority of the forests without conspicuous human intervention and management were found to be disease-free, with the exception of only two places, both in Beira Interior province. Furthermore, important Portuguese ecological hotspots were also sampled during this survey, i.e., the Serra de São Mamede Natural Park and Serra da Estrela Natural Park. These zones represent vulnerable ecosystems, which constitute a valuable ecological patrimony in Portugal. Chestnut stands in both areas were found to be extensively affected by chestnut blight.

The results reported here constitute a pioneer and baseline work from which several other studies have been incorporated aiming to 1) Determine the diversity of vegetative compatibility types and mating types of *C. parasitica* in Portugal as well as

the incidence of natural hypovirulence. This information is required to evaluate the potential for biological control of chestnut blight, following strategies implemented in other regions of the world, and constitutes an essential step in order to block the dissemination of the disease; 2) Analyse the genetic diversity of *C. parasitica* in Portugal mainland and archipelagoes to discern as to the phylogenetic history of *C. parasitica* in the country; 3) Assess the presence of the sexual form of the pathogen, the perithecia, elucidating about its reproductive strategy in infected chestnut areas; 4) Ultimately to evaluate the biotic and abiotic factors which may be directly or indirectly involved in the incidence of the disease.

Our results suggest that the current distribution and severity of chestnut blight in Portugal is of serious concern and that it is imperative to pursue the development of a holistic management strategy, capable of blocking the spread of the disease into the last non-affected chestnut stands, and to optimize the management of the currently infected areas.

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CHAPTER II-2

FACTORS INFLUENCING THE INCIDENCE AND SPREAD OF CHESTNUT BLIGHT IN NORTHEASTERN PORTUGAL

FACTORS INFLUENCING THE INCIDENCE AND SPREAD OF CHESTNUT BLIGHT IN NORTHEASTERN PORTUGAL,

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Abstract

The chestnut blight is a disease caused by the fungus *Cryphonectria parasitica*, being one of the most important mortality factors of the sweet chestnut *Castanea sativa* in Portugal, and widespread in all the regions where chestnut is a relevant component of the landscape. The factors involved in the spread and incidence of the disease were studied for one of the most important chestnut areas in the country, located on Trás-os-Montes e Alto Douro, Northeastern Portugal. Selected variables were altitude, soil type, site orientation, diameter at breast height (DBH), location and orientation of cankers in the tree. The incidence of the disease in the study area was estimated to be 40%. The highest proportion of canker was found in trees with a DHB above 30 cm, most frequently on the 31-40cm diameter class, which could be a consequence of the more intensive management to which those trees are submitted. A higher proportion of cankers was found on the South-facing side of the trunks and branches, with no significant relationship between canker incidence and grove site exposition. None of the other factors analyzed were directly correlated with the incidence and frequency of the disease in the region.

Key-words: *Cryphonectria parasitica*, Chestnut blight, *Castanea sativa*

Introduction

The chestnut blight, caused by the fungus *Cryphonectria parasitica* (Murrill) Barr. is one of the most important causes for the decline of the European chestnut *Castanea sativa* Mill (Heiniger and Rigling, 1994; Robin and Heiniger, 2001). The fungus *C. parasitica* was first identified in the United States of America in 1904, when lesions and necrosis were observed in trunk and branches of the American chestnut [*Castanea dentata* (Marsh) Borkh] led to the almost disappearance of this species locally (Anagnostakis, 1987). In Europe, the disease was first reported in Northern Italy in 1938 (Anagnostakis, 1982; Roane et al., 1986; Bisiach, 1992; Robin and Heiniger, 2001), followed by a widespread expansion of the disease, resulting in immense

chestnut areas affected in beginning of the 1950's (Baldaccio and Orsenigo, 1952). In spite of this, the natural occurrence of hypovirulence and the higher tolerance of the European chestnut to the fungus prevented the mortality levels previously observed in the United States of America (Heiniger and Rigling, 1994; Milgroom and Cortesi, 2004).

In Portugal, European chestnut are mostly located in the central and northern eastern parts of the country, where they assume an important role for the environment (being an important wildlife habitat) and the local economy, due to the harvest of fruit and wood (Abreu, 2007). The first report of extensive mortality caused by *C. parasitica* in Portugal was in 1989, in the Trás-os-Montes e Alto Douro province (Abreu, 1992). Afterwards, the fungi appears to have widespread over a short period of time, as several studies have found it on all major chestnut areas of continental Portugal, being nowadays responsible for a significant tree mortality (Abreu, 1992; Machado et al., 2001; Bragança et al., 2005, 2007).

Several studies have shown the existence of a dynamic relationship between abiotic and biotic factors, anthropogenic influences and plant pathogens (Holdenrieder et al., 2004). This study attempts to assess the relationships between incidence of blight disease and the sanitary condition of the *C. castanea* stands, by studying the most important factors involved in the spread and incidence of this disease in Trás-os-Montes e Alto Douro, Northeastern Portugal.

Material and Methods

The study was conducted in Valpaços county, Trás-os-Montes e Alto Douro province, where the presence of *C. parasitica* was first reported in Portugal (Abreu, 1992). This area is included in the geographic region of "Castanha da Padrela", one of the four Protected Designation of Origin (PDO) regions in Portugal (Mantas et al., 1994;Costa et al., 2005). The area is predominantly composed of orchard-like groves (with a tree spacing of 10x10 m), some of which in association with apple trees, horticulture practices and meadows. According to the bioclimatic classification of Thornthwaite and Hare (1955) the climate of the region can be classified as 'humid' (600 to 1200 $mm.yr^{-1}$), with moderate to severe cold winter periods, with a minimum average temperature of 2º C and a frost period which can last four to seven months (Martins, 2004). The region can be included on the 'sub-atlantic'/'atlantic-

mediterranean' eco-region of Albuquerque (1954), with both montane (700 to 1000 *m.s.m*) and sub-montane (400 to 700 *m.s.m*) altitude ranges. Following the WRB classification (FAO, 1998), the dominant soil-types in the wetter areas are cambisols derived mostly from slate, along with umbric leptosols originated from granite or quartzite, with most soils having acid reactions with pH values of 4.5-5.5.

A total of 32 plots (with each plot approx. 1 ha), distributed in four district parish of Valpaços county, were selected. Trees were individually characterized (location, estimated age, dimension, etc) on each plot. Sampling localities were digitally geo-referenced and mapped using GIS analysis tools with ArcView® 3.2 software (Environmental Systems Research Institute, Inc.) In all plots the dominant variety of chestnut tree was 'Judia', and soil was either schist or granite in origin.

In order to assess the percentage of trees infected with the disease a group sampling was performed, with each group (for a total of 32) corresponding to a sampling unit characterized by common tree features. At each sampling unit only 50% of the trees were observed following alternated tree lines. The total proportion of stand trees infected by *C. parasitica* (p_c) was estimated considering the total sampled trees in the group *i* (m_i), the total infected trees in the group *i* (C_i), the total number of groups (n) and the average number of trees by group ($\overline{M}$) (Marques and Fonseca, 2007):

$$p_c = \frac{\sum_{i=1}^{n} C_i}{\sum_{i=1}^{n} m_i},$$

being the variance $\left(S_{pc}^2\right) = \frac{1}{\overline{M}^2} \text{ x } \left(S_C^2\right)$ x *cpf*, where *cpf* is a correction factor for finite populations whenever ≥ 5% of the population was sampled,

and $\left(S_C^2\right) = \frac{\sum_{i=1}^{n} C_i^2 + p_c^2 \sum_{i=1}^{n} m_i^2 - 2p_c \sum_{i=1}^{n} m_i C_i}{n-1}$

For heterogeneous sampling unit size, the *cpf* was estimated as a proportion of the total plot area [(1- (sampled area / total area)], which in this case is equal to 0.5 (*cpf* = 0.5). According with Marques and Fonseca (2007) the Confidence Interval is calculated as IC$p_c = p_c \pm t_{(31,\ 0.05)}$ x $\sqrt{S^2_{pc}}$.

In addition to the total number of chestnut trees at each site, data on the total number of infected trees as well as the proportion of infected trees for each location and the predominant orientation of cankers were collected. In addition soil type, site

orientation, altitude and the distribution of trees according to DBH values (tree diameter at breast height) were also recorded (original data presented on Table 1, Appendix). Six DBH classes were considered (< 10cm, 11-20cm, 21-30cm, 31-40cm, 41-50cm, 51-60cm, >60cm), both for the total number of chestnut trees and the infected trees. Eight cardinal and inter-cardinal directions were recorded for each site location and canker location in the trees (North, East, South, West, Northeast, Southeast, Southwest and Northwest). The location of the cankers on the trees were also registered according to four classes: trunk, branch, branch base insertion and "entire tree". Branch base insertion was considered because the natural fissuring in this zone could be a hypothetical way in for *C. parasitica*. Almost all groves were pure stands of chestnut European tree with the exception of two stands which also had scattered apple trees. Other data concerning cultural characteristics and practices were also studied (variety, graft provenience, type of grafting, pruning periodicity), but since these characters were found to be very homogeneous throughout the different sampling locations they were not included on the current study.

χ^2 test was used to assess statistical differences in the proportion of trees regarding location and orientation of cankers in the tree, as well as soil type and orientation at sample sites. Spearman's rank correlation test was applied to study the relationship between altitude and the degree of incidence of the disease (proportion of infected trees). As the main assumptions for the use of parametric ANOVA tests were not assumed, the non parametrics Kruskal-Wallis and post-hoc multiple comparisons tests were applied to evaluate differences on the proportion of trees with chestnut blight disease among the tree DBH classes. General Regression Models (GRM) technique was used to analyze the effects and interactions of all site variables measured in each site (*i.e.,* environmental factors, soil type, orientation and altitude), as well as the proportion of chestnut trees infected by *C. parasitica* according with tree DBH classes. GRM assumptions were taken in account (*e.g.* homoscedasticity, normality, collinearity, linearity, among others). Variables were transformed whenever necessary (*e.g.* proportion of canker trees transformed to arcsine; see Zar [1984]). All statistical analyses were made using the Statistica 6.1 software (StatSoft, Tulsa, OK, USA), and the significance level for H_o rejection was $\alpha < 0.05$.

Results

Disease incidence

Cankers attributed to *C. parasitica* were found on all the 32 studied plots, with a total of 512 infected trees among a total of 1235 sampled (see Table 1 of the Appendix). The mean proportion of infected trees was 0.415, and considering a 95% of confidence in the inference ($t_{(31,\ 0.05)} = 2.040$) the confidence interval was found to be [0.178-0.651]. Thus the estimated percentage of infected trees in the study area ranged between 17,8% to 65,1% with a level of confidence of 95%.

Location of cankers in the tree

Results on orientation and location of the cankers in the trees are illustrated in Figures 1 and 2, respectively. Excluding the trees where the disease was widespread all over the tree, the χ^2 test showed a significant difference between the presence of the disease on the locations facing South (being the most frequent) and the SE and NW ones (the rarest) ($\chi^2 = 21.780$; df. = 7; $p < 0.05$).

Regarding the location of the cankers on the trees, and excluding the ones with cankers on the three locations simultaneously, *i.e.* branches, branch base insertions and trunk, a significant difference was observed on the canker location according to the χ^2 test ($\chi^2 = 147.704$; df. = 2; $p < 0.001$), with a much higher proportion of cankers detected on the branches.

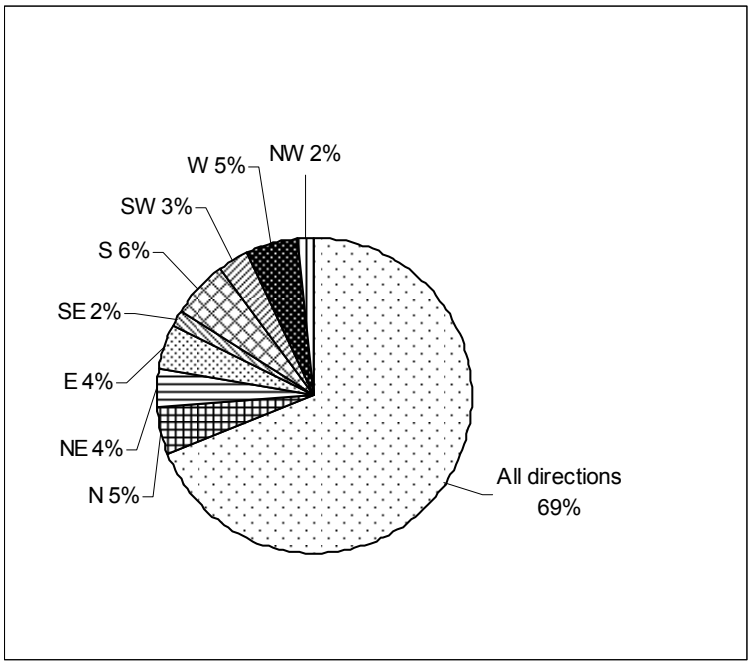

Figure 1 - Affected chestnut trees (%) considering the predominant orientation of *C. parasitica* canker in the tree.

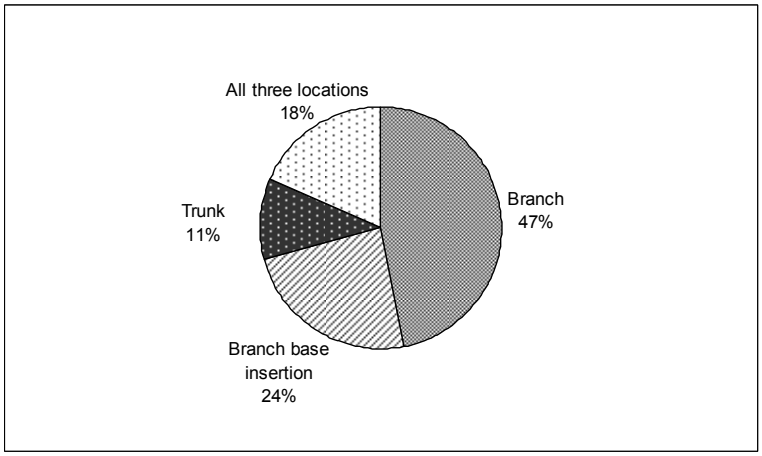

Figure 2 - Affected chestnut trees (%) regarding *C. parasitica* cankers locations in the tree.

Site orientation and soil type effects

Data on the proportion of infected trees regarding site orientation and soil type are presented in Figure 3 and 4, respectively. In both cases the χ^2 tests did not reveal statistical differences in the proportion of infected trees ($p > 0,05$).

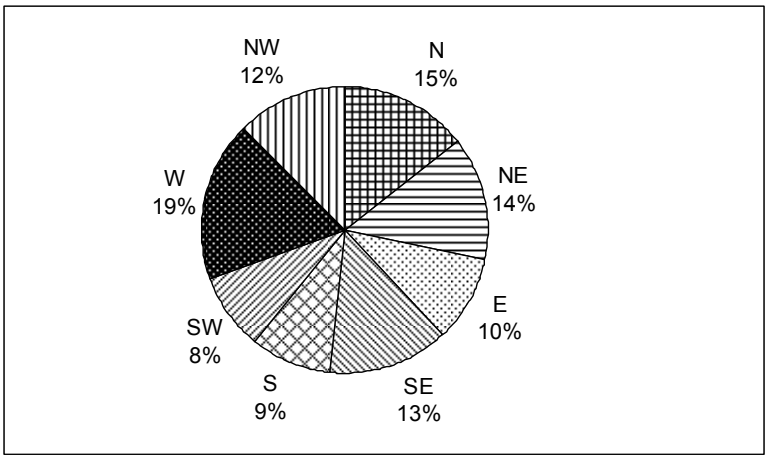

Figure 3 - Chestnut trees infected with *C. parasitica* according to site orientation (%).

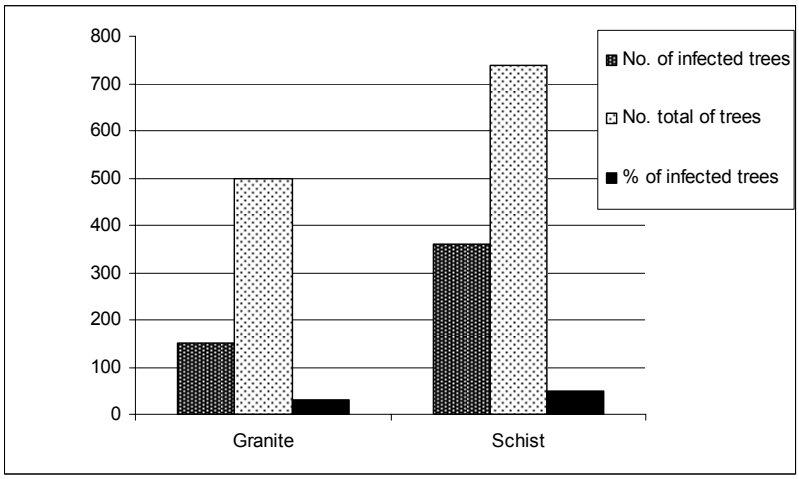

Figure 4 -Number and percentage of chestnut trees with and without *C. parasitica* according to soil type.

Altitude effect

No correlation was found between the altitude of the sites and the proportion of infected trees according to the Spearman's test (r = 0.1400).

Frequency of trees with chestnut blight according to DBH class

Data on the total number of chestnut trees and the number and percentage of infected trees, by DBH class in all sample sites, are shown in Figure 5.

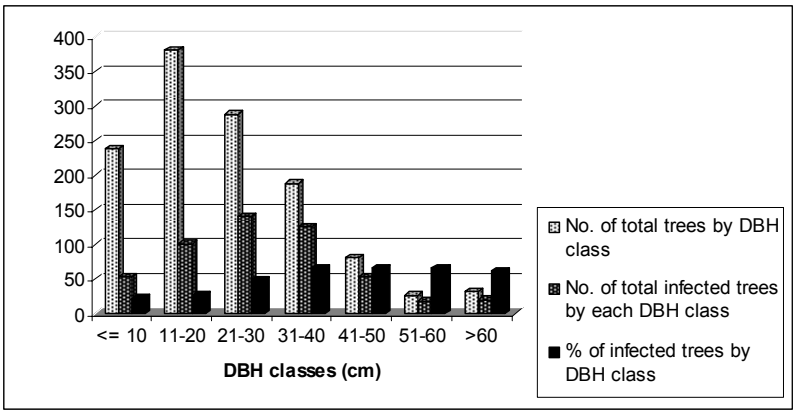

Figure 5 - Number of total chestnut trees and number and percentage of trees infected with *C. parasitica* according to DBH classes at the sampled sites.

The Kruskal-Wallis test revealed significant differences between the incidence of *C. parasitica* and the DBH classes considered ($H_{6,\ 224}$ = 80.787; $p < 0.001$). As summarized in Table 1 post-hoc multiple comparisons confirmed these differences.

Table 1 – Median of the proportion of infected trees among DBH classes

DBH class	Median
<=10 cm	0.210 [a,b]
11-20 cm	0.199 [a]
21-30 cm	0.193 [a]
31-40 cm	0.189 [a,b]
41-50 cm	0.081 [b,c]
51-60 cm	0.036 [c]
>60 cm	0.041 [c]

Medians within the column followed by the same letter do not differ pairwise comparison according with post-hoc Kruskal-Wallis test.

General Regression Model (GRM) of the potential factors influencing the degree of chestnut blight incidence

As previously mentioned on the Material and Methods section, our main aim was to find which external and internal factors in chestnut stands could drive the rate of incidence of *C. parasitica* in these chestnut trees, as well as the possible interactions among them. The potential explanatory variables tested were soil type, orientation and altitude of the sample sites as environmental or extrinsic factors, while tree DBH, being

an approach of the tree age, was tested as an intrinsic factor. The proportion of trees was transformed by arcsine and used as the dependent variable.

The best GRM model we obtained, namely the one generated by forward stepwise method, included only one explanatory variable: the 31-40 cm tree DBH class ($p < 0.001$). This model explains 49% of all variability, and was highly significant (F = 26.177; df. = 60; $p < 0.001$) (see Table 2), with acceptable accomplishment of its main assumptions, namely normality and homoscedasticity. With the exception of tree DBH class 51-60 cm, which was very close to be included in the model (p to enter = 0.068), the other variables and possible interactions where far from that, sustaining all the results of the univariate tests firstly presented.

Table 2 – GRM model summary results.

Test of SS Whole Model vs. SS Residual*						
Dependet Variable	Multiple R	Multiple R^2	Adjusted R^2	SS	Df	MS
ArcSinPArAfe	0.682623	0.465974	0.448173	1.462771	1	1.462771
	SS	df	MS	F	*p*	
	1.676398	30	0.055880	26.17703	0.000017	

Univariate Tests of Significance for ArcSinPArAfe (Regres_AllVar) Final forward stepwise solution Effective hypothesis decomposition					
Effect	SS	Degr. Of Freedom	MS	F	*p*
Intercept	4.436228	1	4.436228	79.38857	0.000000
31-40InftrPr	1.462771	1	1.462771	26.17703	0.000017
Error	1.676398	30	0.055880		

**Multiple R* = coefficient of multiple correlation; *Multiple* R^2 = coefficient of multiple determination; *Adjusted* R^2 = R^2 value, adjusted according with the number of degrees of freedom; *SS* = Sum of the Squares; *Df* = Degrees of freedom; *MS* = Mean Squares (=*SS/Df*); *F* and *p* are the standard (usual) statistics used by the statistical method. *31-40InftrPr* = Effect or variable named "Proportion of infected trees in DBH class of 31-40cm".

Discussion and conclusions

The chestnut blight appears to be widespread through the Valpaços region in Northeastern Portugal, since the fungus *C. parasitica* was found on all the 32 studied plots and our estimate points circa 41% of the chestnut trees attacked by the canker, which can be considered a high infection rate.

Variables such as soil type or site orientation were not found to be correlated with the occurrence of chestnut blight in the Valpaços county, although previously studies have found that cankers are more prevalent in South-facing stands (Martins 2004; Agbaba et al. 2005). The influence of site orientation could be off set by other

environmental, site or management factors in a coarse grain analysis, but our GRM statistical analysis clearly left out from the model the site soil type variable. However, a more detailed analysis at a more fine grain scale – *e.g.* canker orientation at the individual tree -, revealed that the majority of the cankers in the trunk and branches were detected on South-exposed locations. There is no apparent reason to explain this fact, although some authors (*eg,* Gomes-Laranjo et al., 2007) have suggested that South-facing trees may suffer from higher stress, which could lead them to become more susceptible to canker infection.

No relationship was found between altitude and disease incidence, although that could derive from the fact that 75% of the sample sites were located within an altitude range of only 700 - 950 *m.s.m.*.

The low relationship between the degree of canker incidence and the considered environmental factors reinforces the general idea that cultural techniques may be more important to the incidence and dissemination of *C. parasitica* than soil or site characteristics. The higher frequency of cankers in trees with diameters above 30cm could be explained by the higher human intervention on those mature and productive trees, which are subjected to frequent pruning activities and incorrect sanitary operations. Furthermore, the increasing value of the chestnut fruit has resulted in an intensification of the grove exploitation, with frequent soil mobilizations and nitrogen fertilization in addition to the traditional pruning (Abreu and Martins, 2002; Martins and Abreu, 2007). Although other agents may act as dispersal vectors of *C. parasitica*, such as insects and birds (Frigimelica and Faccoli, 1999), pruning is probably the most important factor for the local spreading of the blight. In fact, intensive pruning (to induce fruit production) is a recurring management practice in the local chestnut groves, using hatchets and saws which are seldom disinfected during cutting actions, and therefore can disseminate the chestnut blight through the groves.

Overall, our results suggest that the implementation of good cultural practices may be of fundamental importance to prevent further dissemination and fight the chestnut blight in the North-eastern Portugal. Future studies should focus on the identification and characterization of other abiotic factors on this and other locations, and on clarifying the effective importance of human activities in the spread and incidence of the disease. Nevertheless, the high incidence of the disease and its widespread distribution on the most important chestnut production region of continental Portugal reflect the need for the implementation of effective control actions against this

fungus, which could be a biologic control initiative taking advantage mainly of the presence of hypovirulence (Bragança et al.. 2007) and of a low vegetative compatibility type diversity of *C. parasitica* (Bragança et al. 2005; 2007) which were detected on the region.

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APPENDIX

Table 1. Summary of data collected at each sampling site

Plot code	Nº observed trees	Nº affected trees	% Affected tree	Plot orientation [b]	Soil origin [c]	Altitude (m)	DBH average (cm)	% of tree with chestnut blight in			
								Brunch	Branch bifurcation	Trunk	Entire tree
1	53	8	15	S	G	327	9	25	63	13	0
2	33	9	27	NE	G	846	14	33	33	22	11
3	60	26	43	SE	G	339	31	69	12	12	12
4	20	16	80	N	G	811	36	38	6	6	50
5	49	18	37	SW	SC	941	28	61	17	22	11
6	34	19	56	NE	SC	933	28	42	0	11	47
7	25	15	60	SE	SC	913	30	47	27	13	33
8	19	15	79	SW	SC	935	36	47	33	13	40
9	48	24	50	NE	SC	868	21	50	21	21	29
10	38	17	45	NE	SC	889	35	82	29	0	18
11[a]	45	30	67	N	G	823	34	23	17	0	70
12	51	23	45	S	SC	440	36	70	30	9	4
13	62	34	55	S	SC	516	22	65	79	12	0
14	57	47	82	W	SC	515	35	89	32	4	4
15	46	20	43	N	SC	466	9	5	40	55	5
16	71	4	6	E	G	853	15	50	50	0	0
17	25	10	40	SW	G	847	19	70	30	0	0
18	24	17	71	E	G	341	36	53	35	18	24
19	32	1	3	E	G	875	18	100	0	0	0
20	26	2	8	E	G	491	27	50	0	50	0
21	49	40	82	E	SC	848	16	25	38	20	25
22	22	7	32	SW	SC	733	26	71	71	0	0
23	25	12	48	SE	SC	838	28	83	50	17	0
24	47	6	13	S	SC	807	19	83	17	17	0
25[a]	40	8	20	SW	G	844	33	63	25	25	13
26	64	29	45	E	SC	781	43	72	17	0	21
27	29	9	31	W	SC	798	59	89	0	0	11
28	32	8	25	N	SC	780	32	75	0	25	0
29	13	13	100	NE	SC	870	38	0	0	0	100
30	27	19	70	NW	G	711	37	63	0	5	32
31	42	1	2	SW	G	721	7	0	0	100	0
32	27	5	19	NW	SC	755	25	40	20	40	0
Total	1235	512	41								

[a] Chestnut consociated with apple tree. [b] Cardinal and inter-cardinal directions. [b] G – granite; SC - schist

PART III

GENOMIC DIVERSITY OF *CRYPHONECTRIA* SPP.

CHAPTER III-1

GENOMIC DIVERSITY OF *C. PARASITICA* AND HYPOVIRULENCE - BIOLOGICAL CONTROL PERSPECTIVES

INTRODUCTION

Despite the broad dissemination of the ascomycete fungus *Cryphonectria parasitica* (Murrill) Barr in European chestnut stands since the first identification of the disease in Northern Italy (BIRAGHI, 1946), many chestnut stands began to recover from the disease as indicated by the occurrence of superficial non-lethal chestnut blight cankers. This recovery has been attributed to hypovirulence, a phenomenon mainly caused by the presence of high molecular weight double-stranded (ds) RNAs hypovirus in the cytoplasm and in the mitochondria of the fungus (GRENTE, 1965; VANALFEN *et al.,* 1975; CHOI and NUSS, 1992; BUCK ,1998) but also by mutant forms of mitochondrial DNA (mtDNA) (BAIDYAROY, 2000; BERTRAND, 2000).

The isolates of *C. parasitica* recovered from "healing cankers" containing dsRNA, were named as "hypovirulent" (literally meaning "less virulent") and present abnormal colony morphology and colour, reduced conidiation and reduced virulence (ANAGNOSTAKIS, 1982; HEINIGER and RIGLING, 1994). Through hyphal contact of virulent *C. parasitica* with hypovirulent isolates, the first ones will become hypovirulent by transfer of the dsRNA hypovirus *via* hyphal anastomosis and this process is the basis for biological control (HEINIGER and RIGLING, 1994).

Although hypovirulence has been successfully used for the control of chestnut blight, mainly in therapeutic treatment of individual cankers, biological control has failed almost completely in other cases (MILGROOM and CORTESI, 2004). The success of hypovirulence at the population level depends on the natural spread of dsRNA viruses, which in turn is determined by the interactions between several biotic and abiotic factors (*i.e.* virus, fungus, tree and environment).

The most important viruses for biological control of chestnut blight belong to the family Hypoviridae (HILLMAN and SUZUKI, 2004). Several species of *Cryphonectria* hypoviruses have been described, among them *Cryphonectria* hypovirus 1 (CHV-1), which is widespread throughout Europe and plays a key role in the biological control of the disease (HEINIGER and RIGLING 1994; ALLEMANN *et al.*, 1999; MILGROOM and CORTESI, 2004). Several subtypes of CHV-1 have been detected, varying in virulence to *C. parasitica* (ALLEMANN *et al.* 1999; PEEVER *et al.* 2000).

The hypovirus CHV-1 occurs cytoplasmatically in the fungal mycelium and is transmitted into asexual conidia but not into sexual ascospores (ANAGNOSTAKIS, 1988; PROSPERO *et al.*, 2006). CHV-1 readily spreads via hyphal anastomosis among

C. parasitica isolates with the same vegetative compatibility (vc) type. In contrast, a reduced rate of spread among isolates with different vc type is observed (ANAGNOSTAKIS and DAY, 1979; KUHLMAN *et al.*, 1984; LIU *et al.*, 2003). For different vc type isolates, temporal anastomoses might allow the dsRNA to be transmitted before incompatibility reactions kill the fused cells (HEINIGER and RIGLING 1994).

Vegetative incompatibility or somatic incompatibility is a self/nonself-recognition system in filamentous fungi that has been used to describe fungal population structure and diversity (GLASS and KULDAU, 1992; CORTESI and MILGROOM, 1998). In most ascomycetes, vegetative compability is controlled by allelic interactions in which two individuals are compatible only if they share the same alleles at all *vic* loci (GLASS and KULDAU, 1992; LIU and MILGROOM, 1996). Conversely, individuals are vegetative incompatible when alleles are different at one or more *vic* loci. Thus, vegetative incompatibility phenotypes, referred to as vegetative compatibility (vc) types, are genetically defined by the alleles at multiple *vic* loci, which collectively define the *vic* genotype. The number of known *vic* loci varies among species (CORTESI and MILGROOM, 1998). Most *vic* loci have only two alleles, although multiple alleles have been found in some species *e.g. Neurospora crassa* and *Aspegillus nidulans* (DALES *et al.*, 1993; HOWLETT *et al.*, 1993).

The diversity of vc types (*vic* genotypes) in a population is a function of allelic diversity and recombination among *vic* loci. Because ascomycetes are haploid, 2^n multilocus *vic* genotypes are possible, given two alleles at each of *n* polymorphic, unlinked *vic* loci. Therefore, potential genotypic diversity increases as *n* and allelic diversity increase. If *vic* genotypes were known for vc types, estimates of *n* and allelic diversity would be possible. Furthermore, inferences could be made from vc type survey data about recombination or clonality in natural populations (ANDERSON and KOHN, 1995; LIU and MILGROOM, 1996). In *C. parasitica*, *vic* genotypes for 64 (= 2^6) vc types, controlled by six unlinked *vic* loci (each with two alleles) were determined by CORTESI and MILGROOM (1998) for European population of the fungus. At the population level, high diversity of vc types is believed to be a main obstacle for the success of biological control of chestnut blight (ANAGNOSTAKIS *et al.*, 1986; HEINIGER and RIGLING, 1994; ROBIN *et al.*, 2000; CORTESI *et al.*, 2001). Sexual reproduction is the main source of vc type diversity through the recombination of *vic* genes (CORTESI and MILGROOM, 1998).

Two mating type alleles (*MAT*-1 and *MAT*-2) at a single locus control sexual compatibility in *C. parasitica* (MARRA and MILGROOM, 2001). The mating type ratio in

a *C. parasitica* population provides information about the dominant mode of reproduction (MILGROOM and CORTESI, 1999; HOEGGER *et al.*, 2000; SOTIROVSKI *et al.*, 2004). In sexually reproducing populations, a mating type ratio close to 1:1 is expected. In contrast, deviation from a 1:1 ratio indicates frequent asexual reproduction and favours the spread of the virus that causes hypovirulence. Thus, the population structure, with special regards to vc types and mating types, becomes an important factor for the success of hypovirulence in *C. parasitica* population (BISSEGGER et al, 1997).

There is a considerable variation in vc type diversity among subpopulations in Europe. Low vc type diversity has been observed in areas where the disease has been introduced recently, *e.g.* Germany, Macedonia and Greece (ROBIN and HEINIGER, 2001; SOTIROVSKI *et al.*, 2004; PERLEROU and DIAMANDIS, 2006), or where there is limited sexual reproduction and/or low *vic*-allele diversity (MILGROOM and CORTESI, 1999; HOEGGER *et al.*, 2000). In contrast, relative high vc type diversity has been reported from areas where chestnut blight has been present for a longer period of time, *e.g.* France, Italy and Switzerland, and where sexual reproduction is more frequent (BISSEGGER *et al.* 1997; CORTESI *et al.* 1996; ROBIN et al 2000). In addition, there is a distinct geographic distribution pattern of vc types in Europe with specific vc types being frequent in some areas but rare or absent in other areas (ROBIN and HEINIGER, 2001). For example, EU-12 is the dominant vc type in Southern Italy, Greece, and Eastern Europe, whereas EU-1, EU-2 and EU-5 are dominant in Northern Italy, Southern France, Switzerland and Eastern Spain (ROBIN and HEINIGER, 2001).

Genetic diversity in *C. parasitica* populations has been investigated using classical methodology but also molecular based methods. DNA fingerprinting, through the use of restriction fragment length polymorphisms (RFLPs) and random amplification of polymorphic DNA (RAPDs), have evidenced considerable potential for genetic diversity and fingerprinting of microorganisms (MEYER, *et al.* 1993) and, in particular, for the analysis of the population structure of *C. parasitica* (Milgroom *et al.* 1992,1996). More recently, micro/minisatellite primed polymerase chain reactions (MSP-PCR) have been used to identify genetic variability among closely related vertebrates and other animals, as well as among plants and fungi (MEYER, *et al.* 1993; SAMPAIO *et al.* 2001; GADANHO *et al.* 2003).

In conventional DNA fingerprinting, minisatellite DNA (sequences of repeated motifs of ca. 15 to 30 base pair arranged in tandem at various loci) or microsatellite

DNA (2 to 10 bp arranged in tandem) are detected with hybridization probes. These probes can be used as single primers in the polymerase chain reaction (PCR) to generate individual fingerprints (MEYER, *et al.* 1993). Combining both DNA fingerprinting and RAPD techniques, MEYER *et al.* (1993) have used phage M13 sequences (detects minisatellite DNA), and synthetic oligonucleotides $(GTG)_5$ and $(GACA)_4$, for fast and efficient detection of polymorphic DNA.

The present chapter had three main objectives. First, we used both classic methodology for vc type determination (ANAGNOSTAKIS, 1988; BISSEGGER *et al.*, 1997; CORTESI et al., 1998) and DNA fingerprinting based methods for the detection of hypervariable repetitive DNA sequences (minisatellite or microsatellite DNA) to determine the genetic diversity of *C. parasitica* in Portugal. Second, we estimated the incidence of cankers with perithecia and the mating type ratio in Portuguese subpopulations of *C. parasitica* to assess the mode of reproduction (sexual vs. asexual) of the pathogen. And third, we used culture morphology and dsRNA extractions to screen for the occurrence of hypoviruses in *C. parasitica* from Portugal.

DIVERSITY OF VEGETATIVE COMPATIBILITY (VC) TYPES - PAIRING TESTS AND PCR BASED FINGERPRINT METHODS

Materials and Methods

- **Vegetative compatibility (vc) types**

All 617 Portuguese C. parasitica isolates assessed for vc type were obtained with methodologies previously described in Chapter II -1. From all the area surveyed for the presence of chestnut blight in the course of a nationwide monitoring program, *C. parasitica* was isolated from 108 sites (mainly orchards) across four mainland provinces, Minho, Trás-os-Montes e Alto Douro, Beira Interior and Alentejo; the Azores Islands S. Miguel, Terceira and Pico (Região Autónoma dos Açores), and the Madeira Island (Região Autónoma da Madeira). Considering the importance of the chestnut area and the incidence of chestnut blight in Trás-os-Montes e Alto Douro, this province was divided in 10 zones, resulting in a total of 17 subpopulations (Figure 1).

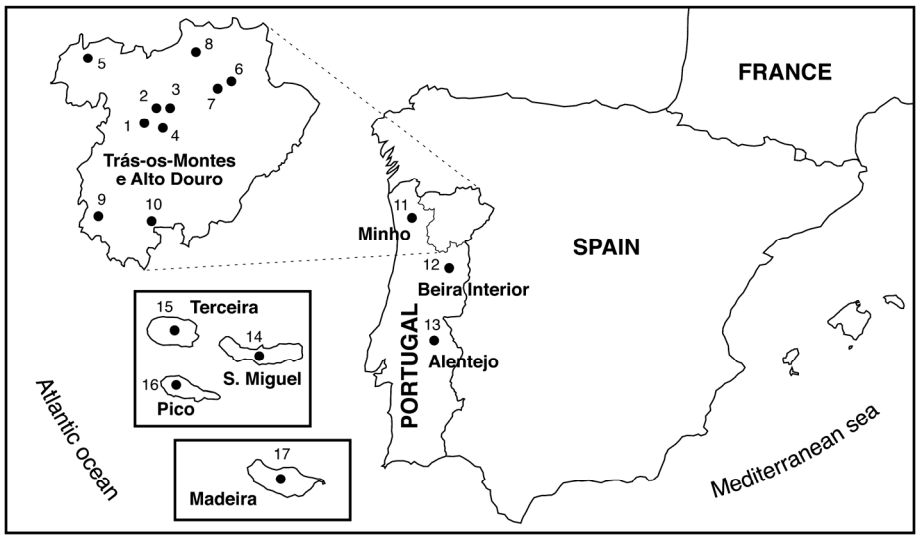

Figure 1- Sampling locations for *Cryphonectria parasitica* in Portugal. Small caps in black colour indicate province names. Numbers indicates subpopulations as defined in Table 1.

The vc type of the *C. parasitica* isolates was assessed according to the barrage/merging response (ANAGNOSTAKIS, 1988; BISSEGGER *et al.*, 1997; CORTESI et al*.,* 1998). Isolates were paired approx. 3 mm apart on potato dextrose agar (PDA; Difco, Sparks, MD, USA) plates and incubated at 25ºC in dark for seven days followed by five days under daylight on the laboratory bench. Six pairing at the edge of an agar Petri dish (diameter 9 cm) was performed (each isolate with itself and five other isolates in each Petri dish) (Figure 2). Petri dishes were inoculated with conidia using a sterile

toothpick, which was scratched over the surface of a sporulating culture. Responses were assessed after seven and 12 days (merging of the two colonies - compatible isolates; barrage between the two colonies - incompatible isolates).

Isolates were initially paired with previously selected Portuguese vc type testers (MACHADO *et al.*, 2001). Isolates incompatible with these testers were paired in all possible combinations, in order to identify new Portuguese vc types. The Portuguese vc types identified were then paired with European vc type testers EU-1 to EU-74 (CORTESI and MILGROOM, 1998; ROBIN *et al.*, 2000).

Figure 2 – Pairings among *Cryphonectria parasitica* isolates on PDA to find vegetative compatibility.

- **MSP-PCR fingerprinting**

A sample of 152 isolates selected from all *C. parasitica* Portuguese subpopulations was assayed by MSP-PCR fingerprinting.

For DNA extraction, mycelium was grown on PDA (Difco) plates covered with cellophane for 7 days at 25ºC in dark. A square of fresh culture (2 x 2 cm) was stripped from the cellophane overlay, transferred to a 2 ml Eppendorf tube, suspended in 500 µl lysing buffer (50 mM Tris ; 250 mM NaCl; 50 mM EDTA , 0.3% w/v SDS; pH 8.0) and the equivalent of 200 µl glass beads - 450/600 µm (Sigma Co., St Louis, MO, USA) was added. After vortexing for 2 min, the tubes were incubated for 1h at 65ºC. The suspension was again vortexed for 2 min and then centrifuged (18000g) for 10 min at 4ºC (adapted from SAMPAIO *et al.* 2001). The supernatant was treated with RNase (50 µg/ml) and the DNA was purified by chloroform/isoamyl alcohol extraction and ethanol precipitation according to PITCHER *et al.* (1989).

The DNA was re-suspended in 50 µl of TE (10 mM Tris, HCl, 2 mM EDTA, pH 8.0) and stored at 4ºC until use.

In this work three primers (Life Technologies, England) were used in the PCR experiments: one minisatellite, a core sequence of the phage M13 (5´-GAGGGGGCGGTTCT-3´) and the two synthetic oligonucleotides $(GACA)_4$ 5′-GACAGACAGACAGACA-3′ and $(GTG)_5$ (5´-GTGGTGGTGGTGGTG-3´) (MEYER *et al.* 1993). All PCR reactions were performed in 25 μL Eppendorf tubes containing approximately 200 ng of template DNA, 1 U of *Taq* DNA polymerase (Life Technologies, England), 50 pmol of each primer (Life Technologies, England), 1x PCR buffer supplied with the enzyme, 2.5 mM $MgCl_2$ and 0.1 mM of each dNTP (Life Technologies, England). To each PCR tube 50 μL of mineral oil were added and amplification was performed in a RoboCycler 96 (Stratagene, La Jolla, CA, USA), according to the following amplification program: 5 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C; and a final step of 5 min at 72°C.

Amplified DNA fragments were separated by electrophoreses in 1 % (w/v) agarose gels (Invitrogen) at 90 V for 3h using the 1kb plus DNA Ladder (Invitrogen) as molecular size marker. After ethidium bromide staining DNA banding patterns were visualized under UV transillumination and images were acquired using KODAK 1D 3.5.2 software.

- **Analysis of genotypic diversity**

The diversity of vc types in each subpopulation was assessed using the Shannon & Wiener diversity H' index, which is calculated with the following expression:

$$H' = -\sum_{I=1}^{S} p_i \ln p_i$$

where p_i is the frequency of the i_{th} vc type in each population. Vegetative compatibility type frequencies were compared among populations using a Chi square test for homogeneity (CORTESI *et al.*, 1996).

Since sample size (number of isolates) varied among subpopulations, we used the rarefaction curve method for estimating Richness (S_{Exp}) and Diversity (H'_{Exp}) amid both Portuguese and all available European populations (LUDWIG and REYNOLDS, 1988). Calculations were made with EcoSim7 software (GOTELLI and ENTSMINGER, 2001), considering 10 000 iterations and the smallest sample sizes of 33 isolates for European populations comparisons, including Portugal. Differences in the expected diversity index (H'_{Exp33}) among European populations were tested with One-way ANOVA and the post-hoc Tukey HSD tests using the Statistica 6.1 software (StatSoft,

Tulsa, OK, USA). The Italian population was subdivided according to CORTESI *et al.* (1996) into two groups - Northern (comprising Donnaz, Crevoladossola, Valtellina, Bergamo, Pigna, Corniglio, Pomino) and Southern Italy (Teano, Cittanova, Zafferana). All ANOVA assumptions were met in this case.

Differences among Portuguese subpopulations were tested by comparing the structure of vc types population for each location (*i.e.* the number of vc types and number of isolates of each vc type), using the Kruskal-Wallis test in the software StatXact 5® (Cytel., Cambridge, MA, USA).

Based on the known *vic* genotypes of the vc types EU-1 to EU-64 (CORTESI and MILGROOM, 1998), allelic diversity at six *vic* loci was calculated for the Portuguese *C. parasitica* populations as described by MILGROOM and CORTESI (1999).

Results

- **Vegetative compatibility (vc) types**

Nine vc types were identified among the 617 Portuguese *C. parasitica* isolates (Table 1; Figures 3 and 4). Seven vc types could be assigned to a known European vc type whereas two vc types were new (P-7 and P-9). P-7 was weakly incompatible with EU-33 and P-9 with EU-70 and EU-74. The dominant and most widespread vc type was EU-11, which comprised 80.2% of all the isolates and was present in all infested provinces (Table 1; Figure 1). EU-12 represented 7.1% and EU-66 6.6% of all isolates. These vc types always co-occurred with EU-11. Four vc types were represented by only one (EU-2, P-7) or a few isolates (EU-1, EU-33). Two vc types, EU-28 and P-9 were only found in Madeira and the Azores archipelagos but not on the mainland. EU-28 and P-9 co-occurred with EU-11 on these Islands.

Table 1 - Vegetative compatibility types of *Cryphonectria parasitica* isolates from Portugal.

Subpopulation	Year	No. of sites	No. of isolates	Vegetative compatibility type								
				EU-1	EU-2	EU-11	EU-12	EU-28	EU-33	EU-66	P-7	P-9
Trás-os-Montes e Alto Douro [a]												
1- Serapicos/S.R.Alharis	2002	9	50			43				7		
2 –S.J.Corveira/Padrela	2002/2005	17	119			111	3			5		
3 –Corveira/Argemil	2002/2003	4	90	2		83	3			2		
4 –Carrazedo Montenegro	2003	6	28	1		8	9			10		
5 -Chaves	2002	2	13			5				8		
6 -Curoupos	1999/2002/2003	10	43			29	7			6	1	
7 -Espinhoso	2003	5	39			37				2		
8 -Bragança	2003	1	40	3		17	19			1		
9 -Tarouca	1999/2003	3	12			12				0		
10- Penedono	1999	1	2			2						
11-Minho	2003	4	19		1	16	1		1			
12-Beira Interior	1999/2001	6	35			33	2					
13-Alentejo	2001	7	30			29			1			
14-S. Miguel	2003	16	38			35			3			
15-Terceira	2003	12	40			30		10				
16-Pico	2003	2	3			1		2				
17-Madeira	2003	3	16			4						12
Total		108	617	6	1	495	44	12	5	41	1	12

[a] Trás-os-Montes e Alto Douro Province was divided into 10 subpopulations.

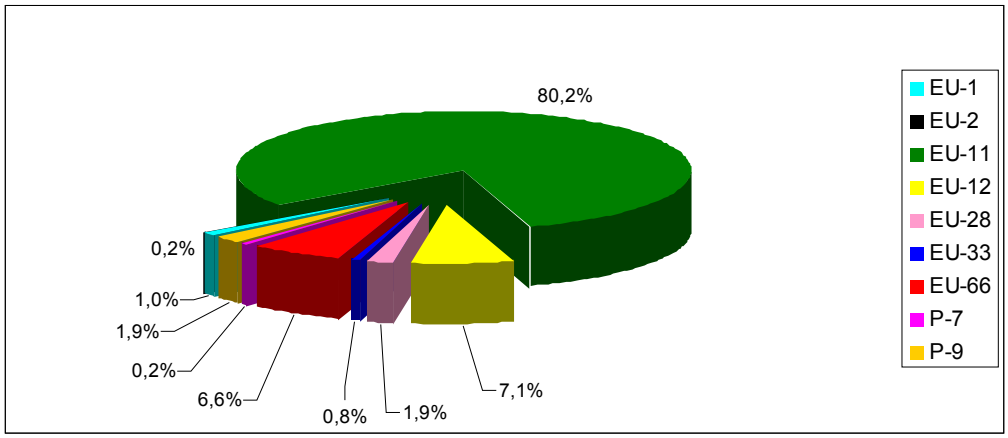

Figure 3 –Vegetative compatibility types % of *Cryphonectria parasitica* isolates in Portugal.

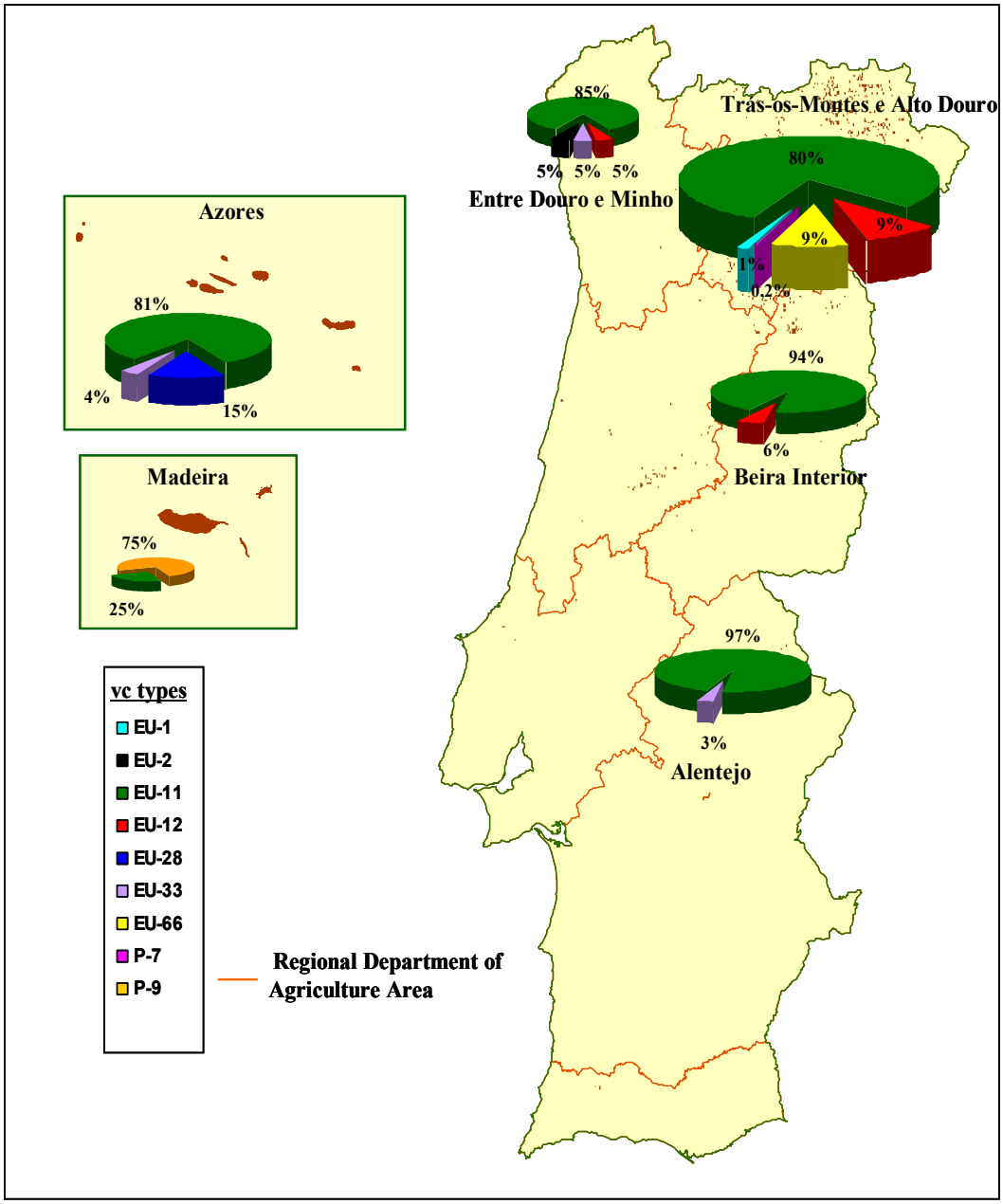

Figure 4 – Distribution of vegetative compatibility types % of *Cryphonectria parasitica* isolates by regions in Portugal

- **MSP-PCR fingerprinting**

Using primers M13, $(GACA)_4$ and $(GTG)_5$, identical DNA patterns with each primer were observed for all *C. parasitica* isolates analyzed, revealing the absence of polymorphisms for these DNA fingerprint markers. In Figure 5 is possible to observe an example of PCR fingerprints obtained after amplification of genomic DNA of Portuguese *Cryphonectria parasitica* isolates (from different areas in country and belong to different vc types).

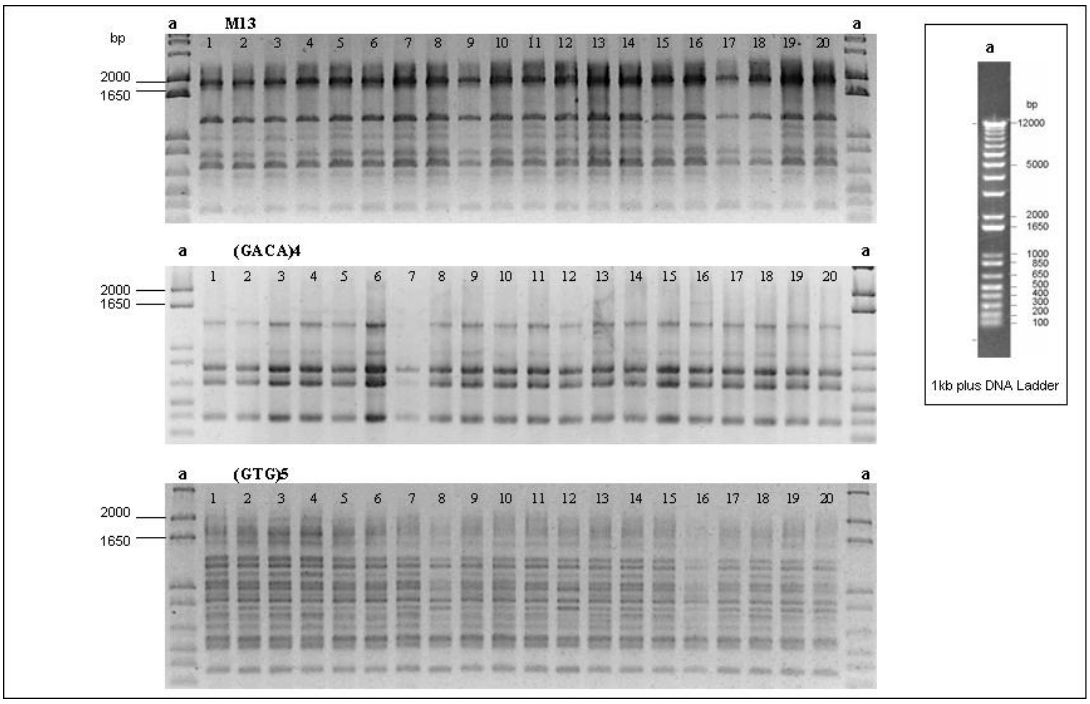

Figure 5 - PCR fingerprints obtained after amplification of genomic DNA of twenty Portuguese *Cryphonectria parasitica* isolates, belong to different vc types, with one minisatellite, phage (M13 core sequence) and two microsatellite probes (GACA)4 and (GTG)5 as single primers. (Lanes: a - molecular size marker 1kb plus DNA Ladder; **1,2,3,4** - Trás-os-Montes e Alto Douro EU-11; **5,6,7,8** - Trás-os-Montes e Alto Douro EU-12; **9** - Minho EU-12; **10, 11, 12** - S. Miguel EU-33; **13** - Minho EU-33; **14, 15** -Trás-os-Montes e Alto Douro EU-1; **16,17, 18, 19, 20** - Terceira EU-28).

- **Analysis of genotypic diversity**

The Kruskal-Wallis test showed that there is no significant differentiation among Portuguese populations in respect to vc types ($T_{(12\ df)} = 4.216$; $p = 0.9792$).

Most Portuguese *C. parasitica* subpopulations had two to four vc types (Tables 1 and 2). The Shannon-Wiener diversity index (H'obs) of these subpopulations varied from 0.146 in Alentejo to 1.209 in Carrazedo Montenegro (Table 2). Most vc types and the highest vc type diversity was observed in Trás-os-Montes e Alto Douro with overall five vc types and H' diversity index values ranging from 1.209 to 0.202.

Table 2 - Richness and diversity of vc types of *Cryphonectria parasitica* in Portugal, Switzerland, Italy and Macedonia.

		Richness		Diversity			
Population	**N**	**S_{Obs}**	**S_{Exp33}**	**H'_{Obs}**	**H'_{Exp33}**	**95% CI**	
Portugal [a]							
Serapicos/S.R.Alharis	**50**	2	2.00	0.405	0.400	0.229	0.517
S.J.Corveira/Padrela	**119**	3	2.43	0.291	0.262	0.000	0.527
Corveira/Argemil	**90**	4	2.96	0.357	0.318	0.000	0.585
Carrazedo Montenegro [b]	**28**	4		1.209			
Curopos	**43**	4	3.77	0.923	0.909	0.760	1.029
Espinhoso	**39**	2	1.98	0.202	0.199	0.136	0.229
Bragança	**40**	4	3.82	1.004	0.993	0.875	1.046
Minho [b]	**19**	4		0.610			
Beira Interior	**35**	2	2.00	0.219	0.218	0.136	0.229
Alentejo [b]	**30**	2		0.146			
S Miguel	**38**	2	2.00	0.276	0.274	0.136	0.305
Terceira	**40**	2	2.00	0.562	0.559	0.474	0.613
Madeira [b]	**16**	2		0.700			
Switzerland [c]							
Lumino	**86**	14	9.28	1.944	1.797	1.484	2.077
Gnosca	**62**	16	11.56	2.181	2.036	1.754	2.303
Italy [d]							
Donnaz	**50**	4	3.66	0.961	0.941	0.765	1.070
Crevoladossola	**131**	10	6.15	1.542	1.432	1.150	1.695
Valtellina	**46**	8	6.79	1.374	1.328	1.093	1.529
Bergamo	**158**	16	7.94	1.761	1.578	1.215	1.916
Pigna	**48**	6	5.05	0.982	0.948	0.714	1.147
Corniglio	**50**	4	3.78	0.816	0.795	0.586	0.971
Pomino	**50**	7	6.10	1.358	1.316	1.081	1.505
Tonara	**33**	5	5.00	0.874	0.874	0.874	0.874
Teano	**194**	8	3.14	0.691	0.619	0.363	0.932
Cittanova	**50**	4	3.32	0.712	0.691	0.501	0.837
Zafferana	**50**	2	2.00	0.405	0.400	0.229	0.517
Macedonia [e]							
Glogi	**46**	4	3.70	0.789	0.773	0.585	0.923
Porogi	**63**	3	2.42	0.272	0.254	0.000	0.437
Vratnica	**49**	2	1.97	0.230	0.225	0.000	0.305
Galate	**57**	3	2.55	0.341	0.323	0.136	0.501
Vrutok	**44**	2	1.75	0.108	0.102	0.000	0.136
Osoj	**72**	3	2.46	0.684	0.664	0.517	0.791

[a] Portuguese data: only Portuguese populations with more then one vc type are shown. [b] Portuguese populations with less then 33 isolates where not included in rarefaction analysis concerning calculations of Richness and Shannon-Wiener H' diversity indexes. [c] Milgroom and Cortesi (1999); Bissegger *et al.* (1997); [d] Cortesi *et al.* (1996); [e] Sotirovski *et al.* (2004); **N** - Number of isolates; **S_{Obs}** - Observed Richness (number of vc types observed); **S_{Exp33}** - Expected Richness for a sample size of 33 isolates; **H'_{Obs}** - H' index observed; **H'_{Exp33}** - Expected H' index for a sample size of 33 isolates; 95% CI - Lower and higher interval limits for 95% of confidence.

Comparing the H' Shannon-Wiener diversity of the population in Portugal with those of other European countries by ANOVA revealed significant differences among the populations (Table 3). The post-hoc Tukey HSD tests showed that vc type diversity in Portugal was comparable to those in Macedonia and Southern Italy ($p>0.05$), but was significantly lower than those in Switzerland and Northern Italy ($p < 0.001$).

Table 3 - Results of one-way ANOVA and Tukey`s HSD tests on H'_{Exp33} values among European populations of *Cryphonectria parasitica*.

	ANOVA				
	SS	df	MS	*F*	p
Intercept [a]	16.345	1	16.345	215.438	<0.001
Group	5.721	4	1.430	18.849	<0.001
Error	1.670	22	0.076		
Tukey HSD tests; Variable H'Exp33					
Error: Between MS = 0.076, df = 22.000					
Group	(1) 0.459	(2) 1.917	(3) 0.390	(4) 1.191	(5) 0.570
(1) Portugal		<0.001 [b]	0.989	<0.001	0.973
(2) Switzerland			<0.001	0.025	<0.001
(3) Macedonia				<0.001	0.885
(4) Northern Italy					0.026
(5) Southern Italy					

[a] Intercept of the regression model [b] p-values for Tukey HSD test

Of the nine vc types found in Portugal, six were among the 64 EU vc types where *vic* genotypes have been determined (CORTESI and MILGROOM, 1998), namely, EU-1, EU-2, EU-11, EU-12, EU-28, and EU-33. Based on these known *vic* genotypes, allelic diversity at six *vic* loci was calculated for five subpopulations with isolates from Trás-os-Montes e Alto Douro and Azores pooled (Table 4). The number of polymorphic *vic* loci range from one in Beira Interior to five in Minho. One *vic* locus (*vic*4) was not polymorphic in Portugal. For the other five *vic* loci, allelic diversity generally was low. Assuming recombination at all known polymorphic *vic* loci, the number of vc types in Portugal could increase to up to 32 vc types.

Table 4 - Allelic diversity at six *vic* loci in Portuguese populations of *Cryphonectria parasitica*.

Population	No. of isolates[a]	*vic 1*	*vic 2*	*vic 3*	*vic 4*	*vic 6*	*vic 7*	Mean	No. of polymorphic *vic* loci	Max. no. of vc types[b]
Trás-os-Montes e Alto Douro	394	0.030	0.187	0	0	0.030	0.030	0.046	4	16
Minho	19	0.199	0.199	0.104	0	0.105	0.105	0.119	5	32
Beira Interior	35	0	0.111	0	0	0	0	0.018	1	2
Alentejo	30	0.067	0	0.067	0	0	0	0.022	2	4
Azores	81	0.228	0	0.072	0	0	0	0.050	2	4

[a] Only isolates with a known *vic* genotype were included in this analysis, i.e. vc types EU-11, EU-1, EU-12, EU-33, EU-28, and EU-2 (Table 1). [b] Maximum number of vc types assuming recombination at all six *vic* loci.

MATING TYPE DETERMINATION AND PRESENCE OF PERITHECIA

Material and Methods

A sample of 152 isolates selected from all Portuguese provinces was assayed for mating type using PCR amplification with the primers M1-GS1 and M1-GS2-rev for *MAT-1* and the primers M2-GS2 and InvA5n for *MAT-2* (MARRA and MILGROOM 1999; MC GUIRE *et al.* 2001). DNA was extracted using the methods described above.
PCR amplification was carried out according to MARRA and MILGROOM (1999) and performed in a T1 Thermocycler Whatman Biometra (Göttingen, Germany)**.** Amplified DNA fragments were separated by electrophoreses in 1 % (w/v) agarose gel at 90 V for 2h using the 1kb plus DNA Ladder (Invitrogen, Carlsbad, CA, USA) as molecular size marker. After ethidium bromide staining, the gels were analyzed with Kodak 1D 3.5.2 software (Kodak, NY, U.S.A.).

All available bark samples stored in EFN laboratory were examined under a dissecting microscope for the presence of perithecia.

Results

Among the 152 isolates tested for mating type, 67 amplified the *MAT-1*(2-kb) and 67 the *MAT-2* (1.7-kb) idiomorph. Eighteen isolates (ten from Terceira, six from S. Miguel, and one each from Pico and Trás-os-Montes e Alto Douro) amplified both *MAT* idiomorphs and were counted for both mating types (see Figure 6 - c, d, g, i, j, k). As shown in Table 5 both mating types of *C. parasitica* were found in all Portuguese provinces.

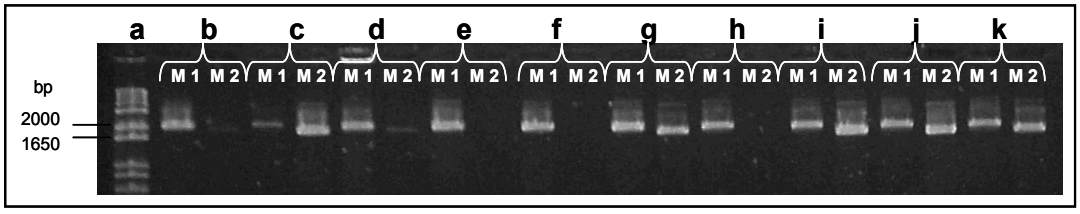

Figure 6 – PCR amplification products of mating type alleles M1 (*MAT-1*) and M2 (*MAT-2*). .Lanes: a - 1-kb plus DNA ladder; b to k - isolates from Terceira Island (Azores).

Table 5 - Mating types of *Cryphonectria parasitica* isolates from Portugal, and incidence of perithecia on the chestnut blight cankers.

Subpopulation	No. of sites	No. of isolates	Mating type [b]		Cankers with perithecia [c]	
			MAT-1	*MAT-2*	n	(%)
Trás-os-Montes e Alto Douro						
1- Serapicos/S.R.Alharis	9	50	0	3	41	17
2 –S.J.Corveira/Padrela [a]	17	119	4	16	84	12
3 –Corveira/Argemil [a]	4	90	8	20	90	27
4 –Carrazedo Montenegro [a]	6	28	9	11	39	36
5 -Chaves	2	13	-	-	7	29
6 –Curoupos	10	43	8	0	-	-
7 –Espinhoso	5	39	0	2	-	-
8 -Bragança	1	40	4	2	-	-
9 -Tarouca	3	12	0	1	-	-
10- Penedono	1	2	-	-	-	-
11-Minho [a]	4	19	3	3	29	31
12-Beira Interior	6	35	3	1	-	-
13-Alentejo	7	30	5	1	-	-
14-S. Miguel [a]	16	38	13	9	47	53
15-Terceira [a]	12	40	21	13	63	54
16-Pico	2	3	1	2	2	50
17-Madeira [a]	3	16	6	1	12	17
Total	108	617	85	85	414	31

[a] Includes cases where both mating types were present within a same site. [b] A subsample of isolates in each populations was assessed for mating types. [c] Percent of cankers with sexual structures (perithecia). n - number of canker samples examined. (-) No data available for these subpopulations.

Using Chi-square statistics, we tested the mating type ratios for deviation from 1:1 in the five subpopulations with large enough mating type frequencies (>5). In two subpopulations (S. J. Corveira/Padrela and Corveira/Argemil), the observed mating type ratio was significantly different from 1:1, whereas in three subpopulations (Carrazedo Montenegro, S. Miguel, and Terceira) the ratio did not differ significantly.

Perithecia were observed at variable frequencies in all ten subpopulations assessed (Figure 7 and Table 5). The incidence of cankers with perithecia ranged from 12% to 54% with a weighted mean of 31%. Perithecia were particularly frequent in populations with a mating-type ratio not different from 1:1, *e.g.* in Carrazedo Montenegro and on the Azores Islands S. Miguel and Terceira.

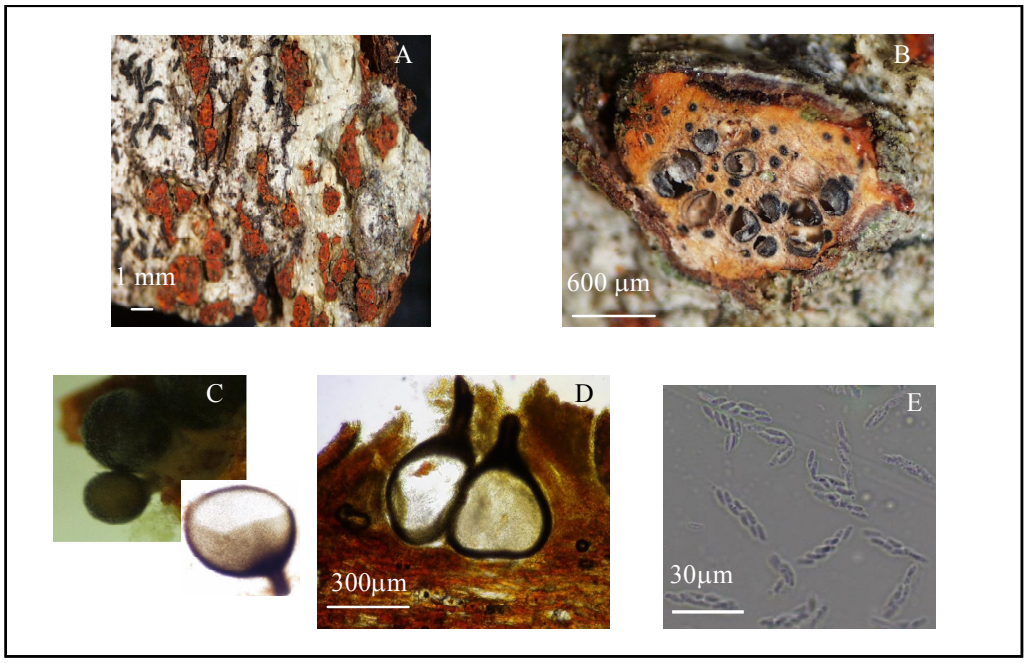

Figure 7 – A - Perithecial stroma of *C. parasitica* emerging through bark of a canker on *C. sativa*; B - tranversal cut of perithecia showing stromata and perithecia necks (dark dots); C and D – intact perithecia; F - asci with 8 ascospores each.

SCREENING FOR dsRNA

Materials and Methods

All *C. parasitica* isolates were tested for hypovirus-infection by assessing their culture morphology. Isolates were grown on PDA plates (Difco) at 25ºC in the dark for seven days, followed by incubation under daylight on the laboratory bench for five days (BISSEGGER et al. 1997). Under these conditions, CHV-1-free isolates produce orange-pigmented cultures with abundant asexual sporulation. In contrast, CHV-1 infected isolates show a white cultural appearance with no or very weak sporulation. A sample of 87 isolates, that showed reduced levels of pigmentation and sporulation was screened for dsRNA, i.e. the genome of the hypovirus. Among the 87 isolates, 56 were from Trás-os-Montes e Alto Douro, 24 from the Azores Islands, five from Alentejo, one from Madeira Island, and one from Minho.

For nucleic acids extraction, mycelium was grown on potato dextrose agar (Difco) plates covered with cellophane for 7 days at 25ºC in dark. The entire *C. parasitica* culture was peel off from cellophane and put in a 2 ml Eppendorf tube. The micelium was lyophilised for 24 hours. The mycelium was mechanically destroyed with a steel ball using a Mikro-Dismembrator (Retsch-Mill - 2min, amplitude 30/sec.) and 40mg of this powder mycelium were used for each isolate. The dsRNA isolations were carried out using cellulose CF-11 chromatography as described by ALLEMANN *et al.* (1999); Nucleic acids were extracted in 2xSTE buffer which contained high salt concentrations and therefore protects the dsRNA from RNase digest. Proteins were precipitated with 10% sodium dodecyl sulfate (SDS), phenol-chloroform and chloroform. The aqueous solution containing DNA, ssRNA and dsRNA was brought to a final concentration of 15% ethanol at which the dsRNA, but not the other nucleic acids, binds to the cellulose CF-11. The cellulose CF-11 was washed with 0,85% 1xSTE/15% ethanol and the dsRNA was eluated with 1xSTE. The dsRNA were precipitated in 0.3 M NaAcetate and 66% ethanol and were collected by centrifugation. The dsRNA preparations were dissolved in RNase-free water and kept at -20ºC.

Since genomic *C. parasitica* DNA (which still can be present in small amount after CF11 chromatography) and CHV-1 dsRNA migrate very similar in agarose gels, dsRNA samples extracted were digested with DNase to confirm their identity. The

Swiss CHV-1 infected *C. parasitica* isolate M784 (ALLEMANN *et al.* 1999) was included as a control in each preparation.

Results

None of the *C. parasitica* isolates sampled before 2003 showed the white culture morphology typical of CHV-1 infected isolates. All isolates exhibited the orange culture morphology, but with some colour variation. Therefore, 87 isolates that appeared somewhat whitish in culture (i.e. showed reduced levels of pigmentation and sporulation on PDA plates compared to the other isolates) were screened for the presence dsRNA. All these isolates proved to be dsRNA-free. In 2005, one single isolate showing white culture morphology was recovered from a healing canker in Padrela, Trás-os-Montes e Alto Douro (Figures 8 and 9).

This isolate belonged to vc type EU-11 and was able to convert compatible orange isolates to white culture morphology. It contained a high molecular-weight dsRNA with the same size (approximately 12 kbp) as the CHV-1 reference isolate. Laboratory pair trials between this EU-11 hypovirulent isolate and isolates from the remaining eight vc types (Figure 10) revealed, in some cases, successful conversion of isolates from incompatible vc types to hypovirulent ones.

Figure 8 – *C. parasitica* culture morphology on Difco Potato Dextrose Agar: virulent isolate presenting the characteristic high levels of sporulation and orange pigmentation (left) and hypovirulent isolate presenting reduced levels of sporulation and pigmentation.

Figure 9 –Healing canker in Padrela,Trás-os-Montes e Alto Douro province (D. Rigling photo).

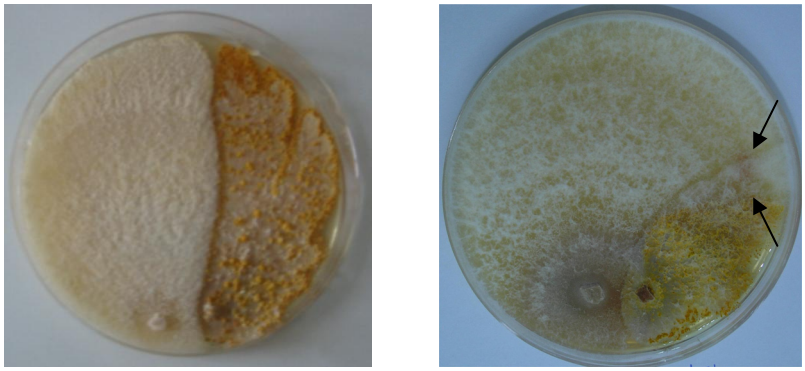

Figure 10 - Pairs of virulent (orange) and hypovirulent (white) isolates of *C. parasitica,* belonging to different vc types, on Difco Potato Dextrose Agar. No conversion of the virulent isolate to a hypovirulent isolate (left); Successful conversion of the virulent isolate to a hypovirulent isolate as indicated by a sector of white converted mycelium (right).

DISCUSSION AND CONCLUSIONS

Our study revealed low vc type diversity of *C. parasitica* in Portugal. The number of vc types identified in each subpopulation ranged from one to four (average 2.6) and the H' diversity indices from 0.146 to 1.209 (average 0.521). In contrast, 14 to 16 vc types have been found in local populations in Southern Switzerland (BISSEGGER *et al.* 1997), four to 16 vc types in Northern Italy (CORTESI *et al.* 1996), and six to 16 vc types in France (BREUILLIN *et al.* 2006). Likewise, H' values of populations in Portugal were significantly lower than those in Southern Switzerland and most populations in Northern Italy (Table 2). However, these comparisons should be made with some cautions because different sampling schemes have been used in different countries. The diversity of vc types in Portugal is comparable to the diversity reported from other peripheral regions where chestnut blight has been established relatively recently such as Hungary (RADÓCZ 2001), Slovakia (JUHÁSOVÁ *et al.* 2005), Switzerland North of the Alps (HOEGGER *et al.* 2000), Germany (SEEMANN *et al.* 2001), Turkey (ÇELIKER and ONOGUR 2001; GURER *et al.* 2001), Macedonia (SOTIROVSKI *et al.* 2004), and Northwestern Spain (AGUIN *et al.* 2005).

The dominant vc type in Portugal was EU-11. This vc type comprised 80.2% of all isolates and was found in all populations across Portugal, including the Islands. Outside Portugal, EU-11 is a very rare vc type that has only been detected in a few locations in Italy (CORTESI *et al.* 1996), France (ROBIN *et al.* 2000), and Hungary (RADÓCZ 2001). The dominance of EU-11 in Portugal might have happened by pure chance as a result of a founder effect. EU-11 has probably been introduced in Trás-os-Montes e Alto Douro where it caused the first disease focus in Portugal. From there it spread throughout Portugal, including the Islands. The other vc types present in Portugal might have been introduced as well (e.g. EU-28 and P-9 on the Azores Islands) or were locally generated by sexual recombination between the introduced vc types.

Among the nine vc types found in Portugal, six were among the 64 vc types with a known *vic* genotype. These six vc types combined were polymorphic at five of the six known *vic* loci. Sexual recombination at all five loci would yield 32 vc types, indicating the potential of an increase in vc type diversity. In addition, we found three vc types with an unknown *vic* genotype, which suggests the presence of an additional polymorphic *vic* locus in Portugal or a third allele at one of the known *vic* loci (ROBIN *et al.* 2000). Either case would provide further potential for an increase in vc type

diversity. vc types with an unknown genotype have also been found in France and Spain (AGUIN *et al.* 2005; ROBIN *et al.* 2000; TRESTIC *et al.* 2001). For example, EU-66, the second most frequent vc type in Portugal has often been found in Dordogne in Western France (ROBIN *et al.* 2000).

The frequent occurrence of perithecia and the mating type ratios observed indicate that sexual reproduction of *C. parasitica* is common in Portugal. In contrast, in Macedonia and Greece, where also one vc type (EU-12) is dominant, *C. parasitica* does not reproduce sexually because of a lack of polymorphism for mating-type (PERLEROU and DIAMANDIS 2006; SOTIROVSKI *et al.* 2004). Similarly, sexual reproduction was also very rare in newly established populations in Switzerland North of the Alps (HOEGGER *et al.* 2000). The frequent sexual reproduction in Portugal is most likely the result of the introduction of isolates of both mating types (probably of EU-11) or isolates that were self-fertile. Self-fertile isolates of *C. parasitica* produce ascospore progeny of both mating types (MCGUIRE *et al.* 2004) and thus can found sexually reproducing populations.

Chestnut blight has spread within less than two decades throughout Portugal, including the Islands. Most likely, sexual reproduction has played an important role in this rapid epidemic through the production of wind-borne ascospores, which can disperse over relatively long-distances (HEALD *et al.* 1915). Transport of infected chestnut plants or wood by people might further account for the rapid spread of chestnut blight in Portugal and is most likely the cause of the infestations on the geographically well-isolated Islands in the Atlantic.

Only one white, hypovirus-infected isolate of *C. parasitica* was found among the 617 isolates tested. This very low incidence of hypovirulence in Portugal could be attributed to a very recent introduction of the hypovirus. The spread of hypovirulence in Europe typically has lagged behind the first observation of chestnut blight (HEINIGER and RIGLING 1994; ROBIN and HEINIGER 2001). In accordance with this finding, the first hypovirus-infected isolate in Portugal was found in Trás-os-Montes e Alto Douro, where chestnut blight was first reported in 1989 (ABREU 1992). The presence of hypovirulence in Portugal offers the potential for biological control of the disease, either naturally or after canker treatments. Whether hypovirulence can establish in Portugal, however, remains to be seen. The low vc type diversity currently observed in Portugal will favour hypovirus invasion of the *C. parasitica* populations. On the other hand, the frequent sexual reproduction could pose a major obstacle to the spread of the hypovirus

because of the potential increase of vc type diversity and the production of hypovirus-free ascospores. At present time, an integrated approach involving the release of hypovirulent isolates and the implement of sanitation measures aiming to remove cankers or dead wood showing signs of the disease, *i.e.* perithecia, might be the best management practice to control chestnut blight in Portugal.

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CHAPTER III-2

CRYPHONECTRIA RADICALIS: REPORT FROM PORTUGAL AND DIAGNOSTIC MOLECULAR MARKERS BASED ON MICROSATELLITE PRIMED PCR

Cryphonectria radicalis: report from Portugal and diagnostic molecular markers based on microsatellite primed PCR

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Abstract

In a recent study intended to assess the distribution of *Cryphonectria parasitica* in Portugal, 22 morphologically atypical orange isolates were collected in the Midwest of Portugal. Eleven isolates were recovered from *Castanea sativa*, in areas heavily affected by chestnut blight and 11 isolates from *Quercus suber* in areas with cork oak decline. To allocate these isolates to the correct taxon, they were compared to known *C. parasitica* and *C. radicalis* isolates using an integrated approach comprising morphological and molecular methods. The morphological features of the atypical isolates were more similar to the *C. radicalis* than to the *C. parasitica* isolates. Phylogenetic analyses grouped the isolates in a separate clade from *C. radicalis sensu stricto* (phylotype I), but together with other isolates considered as *C. radicalis* phylotype II. Genomic fingerprinting of the isolates was performed with microsatellite-primed PCR and diagnostic molecular markers, suitable for the discrimination of *C. radicalis* phylotype I, *C. radicalis* phylotype II and *C. parasitica*, were obtained with $(GACA)_4$ primer.

Keywords: Cork oak tree, Chestnut tree, *Cryphonectria parasitica*, $(GACA)_4$, *Endothiella gyrosa*, MSP-PCR, RFLP-PCR

Introduction

The recently described Cryphonectriaceae family (Gryzenhout et al. 2006a) was defined to accommodate genera from the *Cryphonectria-Endothia* complex (Castlebury et al. 2002). Such genera could be distinguished from those in other families, or undescribed groups in the Diaporthales, by the development of orange stromatic tissue at some stage of their life cycle, a purple reaction in KOH and a yellow reaction in lactic acid associated with pigments in the stromatic tissue or in culture, as well as, a strong phylogenetic support from analysis of ribosomal and β-tubulin genes sequencing (Castlebury et al. 2002).

The new family includes the genera *Cryphonectria* (Sacc.) Sacc. & D. Sacc *Chrysoporthe* Gryzenhout & M.J. Wingf**.,** *Amphilogia* Gryzenh. & M.J. Wingf*.,* *Rostraureum* Gryzenh. & M.J. Wingf*.,* *Microthia* Gryzenh. & M.J. Wingf, *Holocryphia* Gryzenh. & M.J. Wingf., *Endothia* (Schw.) Fr., *Ursicollum* Gryzenh. & M.J. Wingf and *Aurapex* Gryzenhout & M.J. Wingf. (GRYZENHOUT et al. 2005a,b; 2006a,b,c). Some of the most aggressive phytopathogens for forest ecosystems are included among these genera, namely *Cryphonectria parasitica* (Murr.) Barr*.,* *Chrysoporthe cubensis* (Bruner) Gryzenh. & M.J. Wingf (syn. *Chrysoporthe cubensis*), *Endothia. gyrosa* (Schwein.: Fr.) Fr. and *Holocryphia eucalypti* (M. Venter & M.J. Wingf.) Gryzenh. & M.J. Wingf. (syn. *Cryphonectria eucalypti*) (Gryzenhout et al. 2004, 2006a,b,c)

The *Cryphonectria* genus was initially considered a synonym of *Endothia* (Shear et al. 1916, Barr 1978) until the work of Barr in 1978 separated the two genera based on the configuration and texture of the stromata and the septation and shape of the ascospores (Griffin & Elkins 1986, Roane et al. 1986, Myburg et al. 2004b). Nevertheless, species of *Endothia* continue to be mistaken with *Cryphonectria* spp. due to their similar orange fruiting structures and a shared anamorph genus, *Endothiella* (Barr 1978, Gryzenhout et al. 2006b). Moreover, these species share the same hosts (*Castanea* spp. and *Quercus* spp) and geographical distributions (Gryzenhout et al. 2006b). The work of Myburg et al. (1999) showed that *C. parasitica* could be distinguished from *E. gyrosa* using RFLP methods, confirming the species differentiation based on morphological characters (Barr 1978, Venter et al. 2002). Similar results were subsequently achieved in a major study conducted by Myburg et al.

(2004b) intended to clarify the phylogenetic relationships and morphological characteristics of the majority of species in the genera *Cryphonectria* and *Endothia*.

Recent taxonomic revisions restrict the name *Cryphonectria* (*sensu stricto*) to the following species: *C. parasitica*, *C. radicalis* (Schwein.: Fr.) M. E. Barr, *C. macrospora* (Tak. Kobay. & Kaz. Ito) M.E. Barr and *C. nitschkei* (G.H. Otth) M.E. Barr (Gryzenhout et al. 2006a,b,c).

C. parasitica is the most studied fungus among these species since it is the causal agent of chestnut blight disease worldwide, being the only species of this genus to be considered as a primary plant pathogen. This fungus has destroyed nearly all native stands of American chestnuts [*Castanea dentata* (Marsh.) Borkh.]. In Europe, many European chestnuts areas (*Castanea sativa* Mill.) have been similarly affected by the blight in the beginning of the 1930s, although impact and disease severity have been stalled due to natural higher resistance in the European chestnut and the presence of natural hypovirulence (Anagnostakis 1987, Heiniger & Rigling 1994, Bissegger et al. 1997, Milgroom & Cortesi 2004). In the United States and in some European countries, *C. parasitica* has also been found in some species in the genus *Quercus* (Torsello et al. 1994, Radócz & Tarcali 2005), although blight symptoms are not as severe as in *Castanea* spp. (Radócz & Tarcali 2005).

The saprophytic *C. radicalis* occurs in Europe, North America, and Japan, being closely related to *C. parasitica* (Hoegger et al. 2002, Venter et al. 2002, Myburg et al., 2004 a,b). Isolates of *C. radicalis* are difficult to detect in nature because they are possibly almost displaced or their occurrence is cryptically masked by *C. parasitic*a (Hoegger et al. 2002).

In a recent taxonomic review, Gryzenhout et al. (2006b) proposed two different phylogenetic groups of *C. radicalis*, with the first one (*C. radicalis sensu stricto*) defined by the North America type specimen and corresponding morphologically to a group containing isolates from Switzerland, Greece, Italy and Japan (Myburg et al. 2004a), while the second group consisted of isolates from Italy, France and Portugal. These groups are subsequently referred as *C. radicalis* phylotype I and phylotype II, respectively. However, the authors referred, for this species, the inexistence of isolates linked to the original EUA herbarium specimens and because the results of the DNA sequence-based and morphological comparisons cannot be linked, this species has not yet been described as a unique taxon (Gryzenhout et al. 2006b).

In a recent study intended to assess the distribution of *C. parasitica* in Portugal, morphologically atypical orange isolates were unintentionally collected from chestnut stands strongly affected by the blight in the Midwest of the country (Bragança et al. 2007). The different morphology of these isolates was noticed on potato dextrose agar (PDA) medium among *C. parasitica* cultures. A close relationship was established among these and other isolates collected before, from declining *Quercus suber* L., also in the Midwest of Portugal. The culture morphology of these Midwest isolates showed a strong similarity to the culture morphology of an isolate, maintained at the Unidade de Protecção de Plantas collection, and classified (in 1960) as *Endothiella gyrosa* Sacc.. This fungus was first reported in Portugal by Camara (1929) causing the "ferrugem alaranjada" ("orange rust") disease on cork oak stems. However, as previously stated, the difficulty to make correct identifications of these fungi solely based on morphological characteristics, and the absence of the sexual state, makes accurate differentiations difficult.

The patterns of tree decline observed nowadays in Midwest's chestnut and cork oak areas, where the isolates were collected, combined with the information about the known pathogenic behaviour of *Endothiella gyrosa* on *Q. suber* (Oliveira 1931, Natividade 1950) did not allow to reject the hypothesis that these isolates could be a phytopathogenic fungus as *C. parasitica* or *Endothia gyrosa*, since both fungi had been found in *Quercus* spp., namely in *Q. suber* (Venter et al. 2001, Lopez et al.). Nevertheless, Myburg et al. (2004a,b) characterized one isolate, formerly described as *Endothiella gyrosa* (which was collected from *Q. suber* in Portugal by the same author that reported its pathogenicity in *Q. suber*), and reclassified it as *C. radicalis.* Gryzenhout et al. (2006b) grouped this isolate together with one isolate of *C. radicalis* collected in the same host in Italy and genetic evidence showed that the two isolates formed a separate clade from *C. radicalis* phylotype I, with 100% bootstrap support. Based on Myburg et al. (2004a,b) and Gryzenhout et al. (2006a,b), a second hypothesis arose: Do our morphologically atypical isolates represent *C. radicalis*?

Combining the molecular and morphological tools employed by renown important contemporary authors working in this group of fungi (Myburg et al. 1999, 2004a,b, Gryzenhout et al. 2006a, Hoegger et al. 2002) and MSP-PCR fingerprinting method (Meyer et al 1993, Gadanho et al. 2003) the work herein intends to: (1) determine the identity of the isolates reported from Portugal and (2) provide a fast and

reliable methodology to allow discrimination of species or phylotypes within the *Cryphonectria* - *Endothia* or *C. radicalis sensu lato* complexes.

Material and Methods

Fungal isolates

In this study, 22 orange putative *Cryphonectria* spp. isolates from Midwest Portugal (Beira Interior, Ribatejo and Alentejo) were characterized (Table I). These isolates were sampled from *Castanea sativa* bark and *Quercus suber* tissue (mainly collected from cork with exception of C0613 collected from root and C0614 collected from a *Platypus cylindrus* F. insect gallery). All isolates were maintained in a culture collection at the Unidade de Protecção de Plantas, Instituto Nacional de Recursos Biológicos, I.P., Oeiras, Portugal. For comparison, three *C radicalis* isolates from Switzerland (Culture collection of the WSL Swiss Federal Research Institute) and four *C. parasitica* isolates, three from the Portuguese collection and one from the WSL collection, were used.

Culture morphology and growth

The isolates were inoculated on potato dextrose agar (PDA, Difco 39g L^{-1}) Petri dishes and incubated at 25º C in the dark, during one week. The cultures were exposed to diffuse daylight at room temperature on the laboratory bench and culture morphology was observed once a week for a period of four weeks. All cultures were checked for typical *C. radicalis* signs*, i.e.,* purple droplets (under the dissecting microscope) and "flat" center area patterns (Hoegger et al. 2002). Fresh pieces of all fresh cultures (5 x 5 mm) were submersed in parallel into 0.2 ml of 3% of potassium hydroxide and into 0.2 ml of lactic acid to verify purple and yellow change coloration of the mycelium that described by Castlebury et al. (2002).. Spores retrieved from PDA cultures were measured under a light microscope (*Ca.* 10 spores per isolate).

Colony growth measurements were performed based on the methodology described by Hoegger et al. (2002) and Gryzenhout et al. (2004). Cultures were grown on PDA (Difco) for 4 days in the dark at temperatures ranging from 10ºC to 35ºC at 5 ºC intervals. Plates were inoculated by agar discs, 5 mm diam., taken from the edge of

Table1 - List of isolates and respective morphological and molecular techniques applied (*) in this study

Original label of taxon	Final identification	Culture no. [a]	Host	Origin	Collector	Year	Growth in PDA	Corn meal response	PCR-RFLP$_S$	MSP-PCR
Cryphonectria sp.	*C. radicalis* phylotype II	C 0084	*Quercus suber*	Portugal-A	A. Macara	1960	*	*	*	*
Cryphonectria sp.	*C. radicalis* phylotype II	C 0605	*Q. suber*	Portugal-A	H. Bragança & P. Piloto	2001	*	*	*	
Cryphonectria sp.	*C. radicalis* phylotype II	C 0606	*Q. suber*	Portugal-A	H. Bragança & P. Piloto	2001	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0607	*Q. suber*	Portugal-A	H. Bragança & P. Piloto	2001	*	*	*	*
Cryphonectria sp.	*C. radicalis* phylotype II	C 0608	*Q. suber*	Portugal-A	H. Bragança	2000	*	*	*	*
Cryphonectria sp.	*C. radicalis* phylotype II	C 0609	*Q. suber*	Portugal-A	H. Bragança & P. Piloto	2001	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0610	*Q. suber*	Portugal-A	H. Bragança	2000	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0611	*Q. suber*	Portugal-A	H. Bragança & P. Piloto	2001	*	*	*	*
Cryphonectria sp.	*C. radicalis* phylotype II	C 0612	*Q. suber*	Portugal-A	H. Bragança & P. Piloto	2001	*	*	*	*
Cryphonectria sp.	*C. radicalis* phylotype II	C 0613	*Q. suber*	Portugal-R	H. Bragança	2005	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0614	*Q. suber*	Portugal-A	L. Inácio	2005	*	*	*	*
Cryphonectria sp.	*C. radicalis* phylotype II	C 0679	*Castanea sativa*	Portugal-B	H. Bragança	2001	*	*	*	*
Cryphonectria sp.	*C. radicalis* phylotype II	C 0682	*C. sativa*	Portugal-A	H. Bragança & J. Marcelino	2001	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0683	*C. sativa*	Portugal-A	H. Bragança & J. Marcelino	2001	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0684	*C. sativa*	Portugal-A	H. Bragança & J. Marcelino	2001	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0685	*C. sativa*	Portugal-A	H. Bragança & J. Marcelino	2001	*	*	*	*
Cryphonectria sp.	*C. radicalis* phylotype II	C 0686	*C. sativa*	Portugal-A	H. Bragança & J. Marcelino	2001	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0687	*C. sativa*	Portugal-A	H. Bragança & J. Marcelino	2001	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0691	*C. sativa*	Portugal-A	H. Bragança & J. Marcelino	2001	*	*	*	*
Cryphonectria sp.	*C. radicalis* phylotype II	C 0692	*C. sativa*	Portugal-A	H. Bragança & J. Marcelino	2001	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0704	*C. sativa*	Portugal-A	H. Bragança & J. Marcelino	2001	*	*		
Cryphonectria sp.	*C. radicalis* phylotype II	C 0705	*C. sativa*	Portugal-A	H. Bragança & J. Marcelino	2001	*	*	*	*
C. radicalis	*C. radicalis*	CM2269[b]	*C. sativa*	Switzerland	P. Hoegger	1995	*	*	*	*
C. radicalis	*C. radicalis*	CM2270[b]	*C. sativa*	Switzerland	D. Rigling	1996	*	*	*	*
C. radicalis	*C. radicalis*	CM4733[b]	*C. sativa*	Switzerland	D. Rigling	2005	*	*		*
C. parasitica	*C. parasitica*	CEP155[b]	*C. dentata*	USA	S. Anagnostakis	1977	*	*	*	*
C. parasitica	*C. parasitica*	C 0720	*C. sativa*	Portugal-TM	H. Bragança	2002	*	*	*	*
C. parasitica	*C. parasitica*	C 0721	*C. sativa*	Portugal-TM	H. Bragança	2002	*	*	*	*
C. parasitica	*C. parasitica*	C 0722	*C. sativa*	Portugal-T	H. Bragança	2003	*	*	*	*

[a] Culture collections of the Unidade de Protecção de Plantas, Instituto Nacional de Recursos Biológicos, I.P., Oeiras ("C" refers *Cryphonectria* culture collections). [b] Includes original reference. For Portugal, the capital letter states the province: A –Alentejo, B – Beira Interior, Ribatejo e Oeste, TM – Trás-os-Montes e Alto Douro, T – Terceira.

actively growing cultures, and placed in the centre of the 90mm diam Petri dish. The perpendicular diameters (at quadrant N-S and E-W) of each culture were measured.

In addition, isolates were grown on corn meal medium, prepared as described by Hoegger et al. (2002), adding 10g of corn meal to 20mL of distilled water in 100 mL Erlenmeyer flasks (autoclaving 20 min at 120ºC). The cultures (two per isolate) were incubated at 25ºC in the dark and assessed for change in culture pigmentation after seven weeks.

DNA extractions

For DNA extraction, mycelium was grown on PDA Petri dishes covered with cellophane for 7 days at 25ºC in dark. A square of fresh cultures (2 cm x 2 cm) was stripped from the cellophane overlays, transferred to a 2 ml Eppendorf tube and the extraction was performed using Raeder & Broda (1985) methodology. First, the mycelium was suspended in 500 µl lysing buffer (300 mM Tris-HCl, pH 8.5 ; 250 mM NaCl; 25 mM EDTA, 0.5% w/v SDS) and the equivalent of 200 µl of 450-600 µm glass beads (Sigma) was added. After vortexing for 2 min, the tubes were centrifuged (max V.) for 10min at 4ºC (adapted from Sampaio et al. 2001). The supernatant was mixed with 350 µl of phenol and 150 µl chloroform and centrifuged for 15 min (max V.). The following steps were done exactly as described in Raeder & Broda (1985). The DNA was resuspended in 50 µl of TE (10 mM Tris - HCl, 2mM EDTA, pH 8.0) and stored at 4ºC until use.

Ribosomal DNA (ITS1-5.8S-ITS2) amplification and PCR - RFLPs

The internal transcribed spacer region, ITS (which includes the 5.8S rDNA gene) was amplified by PCR using forward primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') described by White et al. (1990). The PCR reaction mixture (50 µL) included 400 ng of template DNA, 2 U of Taq DNA polymerase (Life Technologies, England), 50 pmol of each primer (Life Technologies, England), 1 x PCR buffer supplied with the enzyme, 4 mM $MgCl_2$ and 200 µM of each

dNTP (Life Technologies, England). To each PCR tube 50 μL of mineral oil were added and amplification occurred in a RoboCycler 96 (Stratagene, La Jolla, CA, USA), according to the following amplification program: 4 min at 95°C; 30 cycles of 1min at 95°C, 1 min at 56°C and 1 min at 72°C; 5 min at 72°C.

Based on Myburg et al. (1999) a restriction analysis of ITS region (ITS1, 5.8S, ITS2) was performed. From each PCR product 5 μL samples were digested with 3 U of restriction endonuclease *Alu*I (New England Biolabs, Beverly, MA, USA), in a final volume of 10 μL, according to manufacturer's instructions. The digested PCR products were separated on 2% w/v agarose gel (Invitrogen) in 0.5 x TBE (50 mM Tris, 45 mM boric acid, 1 mM EDTA) at 90 V for 3 h, using 100 bp DNA Ladder (Gibco-BRL) as a molecular size marker. After ethidium bromide staining, the gels were analysed with KODAK 1D 3.5.2 software.

β-tubulin gene amplification

Two regions within the β-tubulin gene were amplified using primer pairs Bt1a, Bt1b (amplifying β-tubulin region 1) and Bt2a, Bt2b (amplifying β-tubulin region 2) (Glass and Donaldson 1995). The amplification reaction mixture and reaction conditions were as described by Myburg et al. (2002).

Each reaction sample was run on a 1.2% w/v agarose gel (Invitrogen), in 0.5 x TBE (50 mM Tris, 45mM boric acid, 1mM EDTA) at 90 V for 2h, using 100 bp DNA Ladder (Gibco-BRL) as molecular size marker. After ethidium bromide staining, the gels were analysed with KODAK 1D 3.5.2 software.

Ribosomal DNA (ITS1-5.8S-ITS2) and β-tubulin gene sequencing - phylogeny reconstruction

All sequences were obtained in an automated DNA capillary sequencer CEQ 2000-XL (Beckman Coulter, USA, in ICAT-Lisbon Faculty of Sciences Sequencing Services) by a dye-labeled dideoxy termination method (DTCS, Dye Terminator Cycle sequencer start kit, Beckman Coulter). For sequencing, the PCR products were purified using Jet Quick-PCR Purification Kit (Genomed) as described by the manufacturer. Two sequencing reactions were performed for each primer pair used in PCR

amplification. For each isolate, the partial sequence was assembled combining the sequences generated by each primer, using the CEQ Investigator program (software CEQ 8000, Beckman Coulter).

Phylogenetic analyses were conducted in MEGA4 software (Tamura et al., 2007). Nucleotide sequences from ribosomal (ITS1, 5.8S, ITS2) and β-tubulin genes (1a/b, 2a/b) were aligned with ClustalW default settings (Higgings et al., 1994) and all positions containing gaps and missing data were eliminated. A combined data set was also generated by concatenation of aligned matrices. Genetic distances were computed using the Kimura 2-parameter model with a transition/transversion ratio of 2:1 (Kimura, 1980) and phylogenetic trees were inferred with the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis (Felsenstein, 1985) was performed with 1000 replicates. For all sequences generated in our study, Genbank accession numbers are listed in Table 1. Additional sequence data used in the phylogenetic analyses were obtained from Myburg et al. (2004a,b) and Gryzenhout et al. (2006a). Sequences from *Diaporthe ambigua* isolates were used as outgroup to root the phylogenetic trees.

MSP-PCR fingerprinting

In the PCR fingerprinting method using single micro/minisatellite primers (MSP-PCR) three primers were used: a core sequence of the phage M13 (5′-GAGGGGGCGGTTCT-3′) and the two synthetic oligonucleotides, $(GACA)_4$ 5′-GACAGACAGACAGACA-3′ and $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) (MEYER et al.. 1993). All PCR reactions were performed in 25 μL Eppendorf tubes containing approximately 200 ng of template DNA, 1 U of *Taq* DNA polymerase (Life Technologies, England), 50 pmol of each primer (Life Technologies, England), 1x PCR buffer supplied with the enzyme, 2.5 mM $MgCl_2$ and 0.1 mM of each dNTP (Life Technologies, England). To each PCR tube 50 μL of mineral oil were added and amplification was performed in a RoboCycler 96 (Stratagene, La Jolla, CA, USA), according to the following amplification program: 5 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C; and a final step of 5 min at 72°C.

Amplified DNA fragments were separated by electrophoreses in 1 % (w/v) agarose gels (Invitrogen) at 90 V for 3h using a 1Kb plus DNA Ladder (Invitrogen) as molecular size marker. After ethidium bromide staining, DNA banding patterns were

visualized under UV transillumination and images were acquired using KODAK 1D 3.5.2 software.

Results

Culture morphology on PDA, pigmentation on corn meal medium and growth

After one week, the pinkish feature due to the development of small droplets of purple exudates on the hyphae, visible under the dissecting microscope, was only observed in the three *C. radicalis* isolates. The Portuguese *Cryphonectria* sp. showed orange pigmentation in the center of the culture, with some variations, whereas the three *C. radicalis* remained white. After four weeks under artificial light, it was possible to observe three different types of culture morphologies, associated with Portuguese *Cryphonectria* sp.*, C radicalis*, or *C. parasitia,* respectively. However, as observed in Fig 1, *C. radicalis* and *Cryphonectria* sp. cultures were much closer in morphology. The "flat" center area was present only in *C. radicalis* isolates (Figs 1b). All cultures showed purple and yellow coloration reaction to the KOH and lactic acid. *Cryphonectria* sp. cultures produced the largest conidia in comparison to *C. parasitica* cultures (Fig 2). *Cryphonectria* sp. conidia were found to be irregular in form and size (5.2 - 7.5 X 1.3 - 2.5 μm) (Figs 2a1 and a2). On corn meal all three *C. radicalis* isolates caused a colour change in the medium from natural beige medium to purple (Fig 1e). All *C. parasitica* failed to produce purple pigmentation in the corn meal medium (Fig 1f). The Portuguese *Cryphonectria* sp. cultures presented a reddish-purple pigmentation although not as intense as observed in the *C. radicalis* reference cultures (Fig 1d).

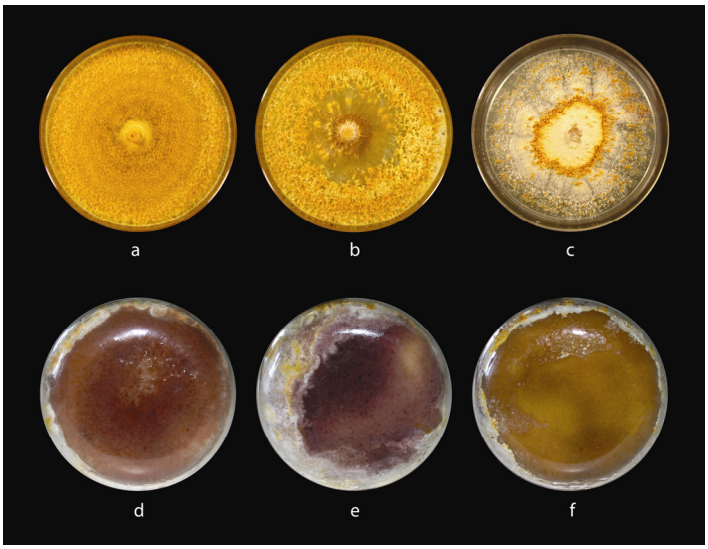

Fig 1 - Culture morphology on potato dextrose agar and corn meal coloration: a and d – Portuguese *Cryphonectria* sp., b and e - *C. radicalis* , c and f– *C. parasitica*.

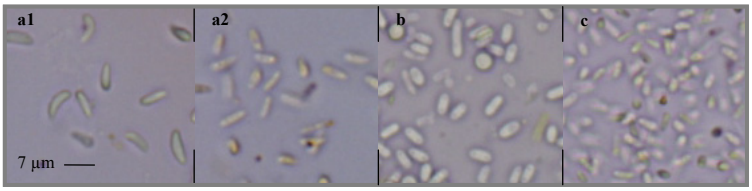

Fig 2 – Conidial spores produced in PDA cultures: a - *Cryphonectria* sp. (a1 and a2 represent different type of spores, b - *C. radicalis*, c- *C. parasitica*.

The mean differences in growth are showed in Fig 3. All cultures grew better at 20 and 25ºC. Isolates of *C. radicalis* showed a higher growth rate, at all temperatures tested, with exception of 30ºC where *C. parasitica* presented the highest growth rates. However, since standard deviations of growth were high no statistical significant association could be found between growth rate and taxonomic groups.

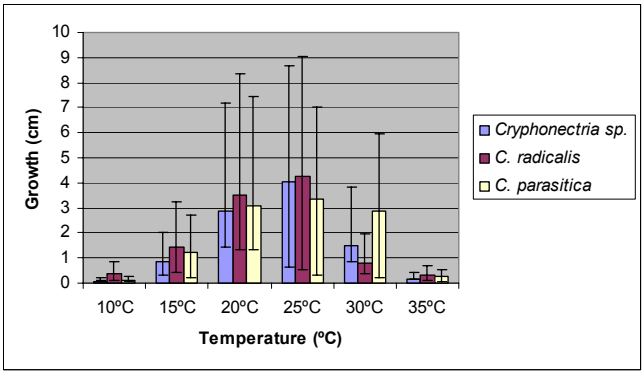

Fig 3 – Colony growth of *Cryhonectria* sp., *C. radicalis* and *C. parasitica* on PDA medium (Mean colony growth measured after four days ± standard deviation, n= 5).

Ribosomal DNA (ITS1-5.8S-ITS2) and β-tubulin gene amplification and PCR - RFLPs

Amplification of the ITS1-5.8S-ITS2 and β-tubulin gene regions resulted in a single fragment of approximately 700 and 600 bp, respectively.

The restriction of the ITS1-5.8S-ITS2 region with enzyme *Alu* I produced a unique RFLP profile for all isolates (two fragments with approximately 475 and 240 bp), which is compatible with the *C. parasitica* profile presented by Myburg et al. (1999) (Fig 4).

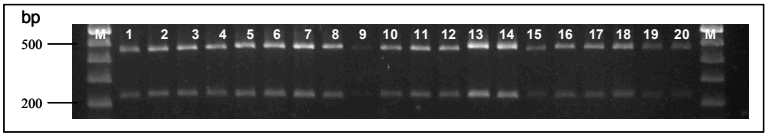

Fig 4 – Agarose gel of the internal transcribed spacer-restriction fragment length polymorphism (RFLP) profiles generated by the restriction of the ITS1-5.8S-ITS2 region with restriction enzyme *Alu* I. Lanes 1 to 4 and 10 to 12 represent *Cryphonectria parasitica* isolates (three Portuguese isolates not referred in Table 1 were include in the gel) , 5 to 9 *Cryphonectria* sp. isolates from *Castanea sativa*, 13 and 14 *C. radicalis* isolates and 15 to 20 *Cryphonectria* sp. isolates from *Quercus suber*. M – molecular size marker (100 bp DNA Ladder).

Phylogenetic allocation of *Cryphonetria* sp. isolates

The phylogenetic trees obtained from the ribosomal sequence data (ITS1, 5.8S, ITS2) and from a concatenated data set of ribosomal (ITS1, 5.8S, ITS2) and ß-tubulin gene (1a/b, 2a/b) sequences are shown in Fig 5 and Fig 6, respectively. Both topologies, using *Diaporthe ambigua* as outgroup, are largely congruent and the phylogenetic relations found amongst *Cryphonectria* spp. and *Endothia* spp. are compatible with the results presented by Myburg et al. (2004a,b) and Gryzenhout et al. (2006a,b). These data confirm two separate clades for *C. radicalis* species: *C. radicalis* phylotype I, considered by Gryzenhout et al. (2006b) *C. radicalis sensu stricto*, that includes two isolates from Switzerland and two from Italy, and *C. radicalis* phylotype II, grouping Portuguese isolates of *Cryphonectria* sp. with another Portuguese isolate and an Italian isolate (100% bootstrap support). As expected, Portuguese *C. parasitica* isolates are in the same clade with the other *C. parasitica* (99% bootstrap support). The final identification of isolates is presented in Table 1.

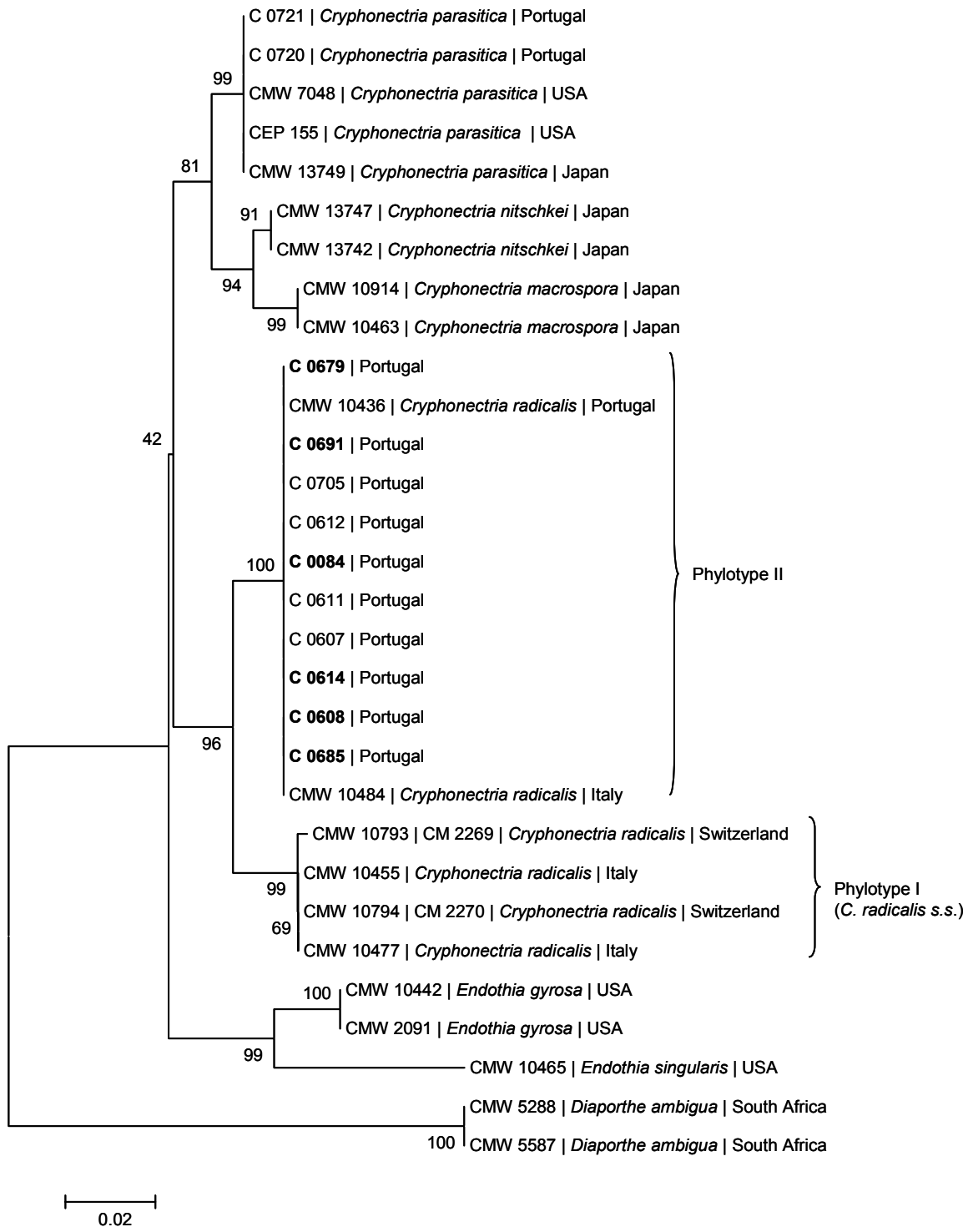

Fig 5 - Neighbour-joining tree obtained from ribosomal sequence data (ITS1, 5.8S, ITS2). Bootstrap values (1000 replicates) are shown next to branch nodes. Phylogenetic distances were computed using the Kimura 2-parameter method (scale: number of base substitutions per site). A total of 438 positions was used in the final dataset, after removing all positions containing gaps and missing data.

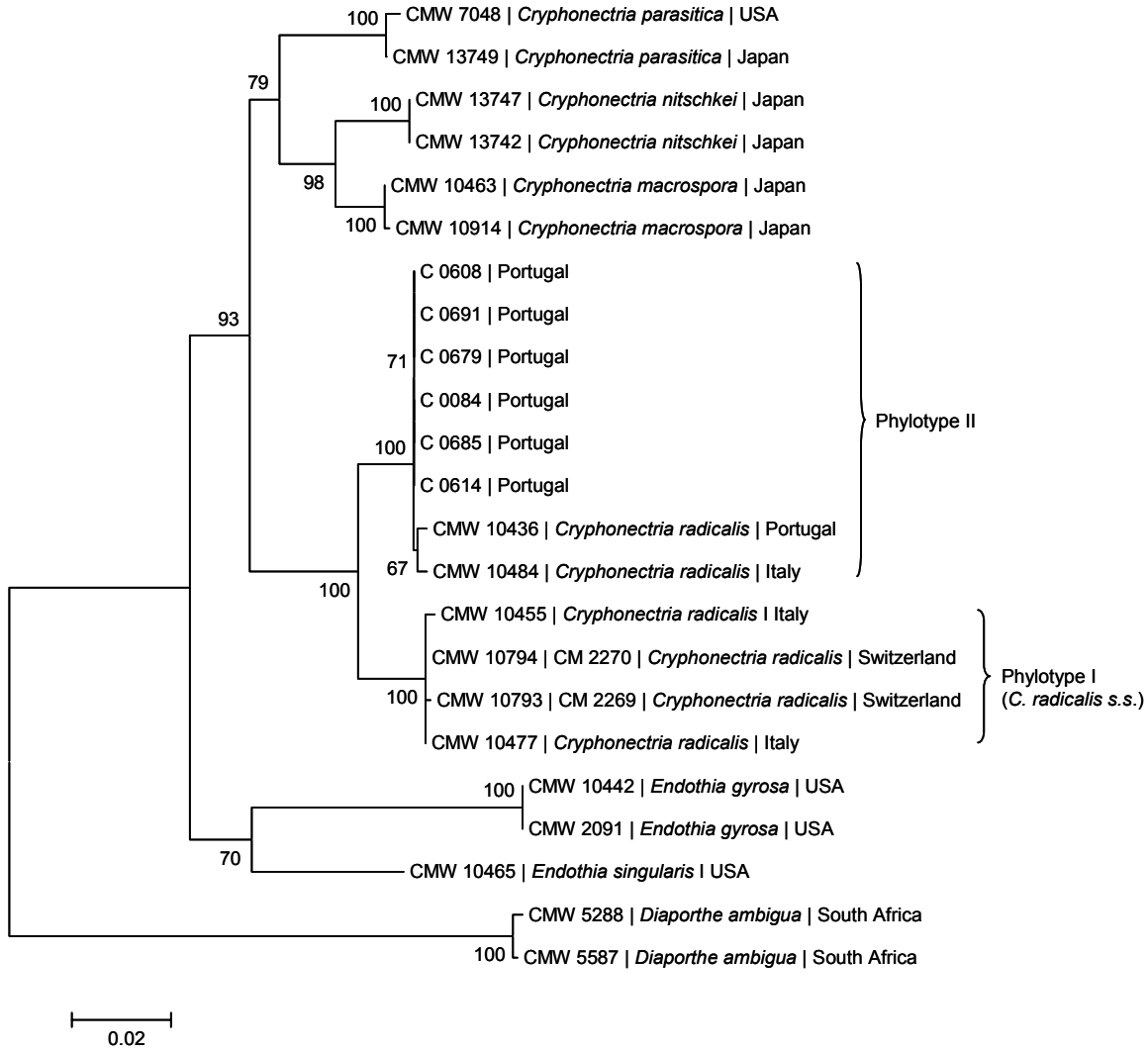

Fig 6 - Neighbour-joining tree obtained from a concatenated data set of ribosomal (ITS1, 5.8S, ITS2) and b-tubulin gene (1a/b, 2a/b) sequences. Bootstrap values (1000 replicates) are shown next to branch nodes. Phylogenetic distances were computed using the Kimura 2-parameter method (scale: number of base substitutions per site). A total of 1068 positions was used in the final dataset, after removing all positions containing gaps and missing data.

MSP-PCR fingerprinting

Distinct electrophoretic band patterns were obtained with M13, $(GTG)_5$, and $(GACA)_4$ primers, with each primer enabling differentiation of all three groups tested, *C. radicalis* phylotype II, *C. radicalis* phylotype I, and *C. parasitica* (Fig 5). Electrophoretic band patterns obtained by $(GACA)_4$ were more evident than M13 and $(GTG)_5$ patterns. The $(GACA)_4$ PCR fingerprints showed well separate main DNA fragments, four associated with *C. radicalis* phylotype II isolates and three with *C. radicalis* phylotype I and *C. parasitica* isolates, which size is presented in Fig 6. Since all Portuguese *Cryphonectria* sp. isolates had the same banding patterns, this method confirmed the *C. radicalis* phylotype II identification for the non-sequenced isolates.

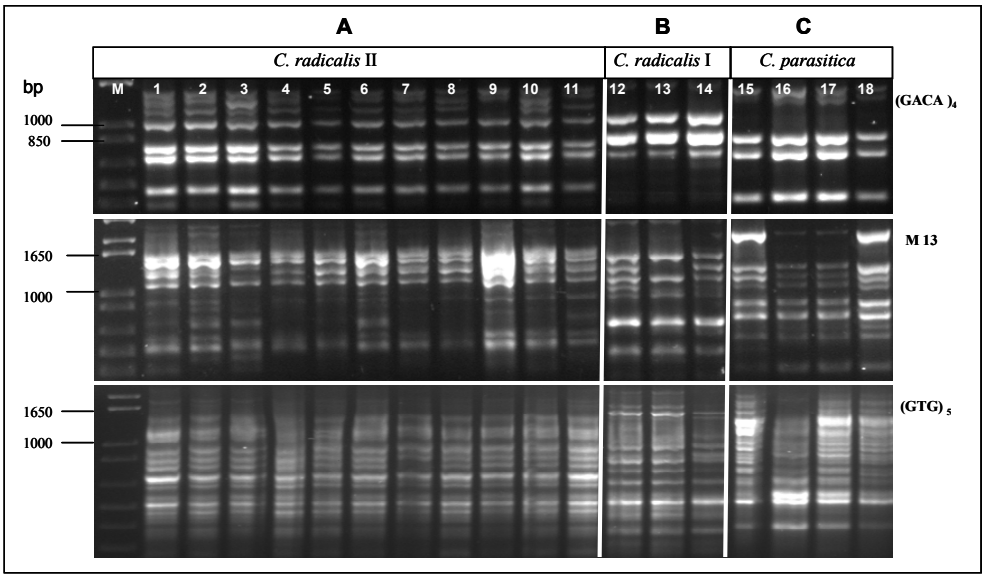

Fig 5 – MSP-PCR profiles of *C. radicalis* phylotype II, *C. radicalis* phylotype I and *C. parasitica* isolates using $(GACA)_4$, M13 and $(GTG)_5$ primers: 1 – C0084, 2 - C0605, 3 - C0607, 4 - C0608, 5 - C0611, 6 - C0612, 7 - C0614, 8 - C0679, 9 - C0685, 10 - C0691, 11 - C0705, 12 - CM2269, 13 - CM2270, 14 - CM4733, 15 - CEP155, 16 - C0720, 17 - C0721, 18 - C0722. M - molecular size marker (1 kb plus DNA Ladder).

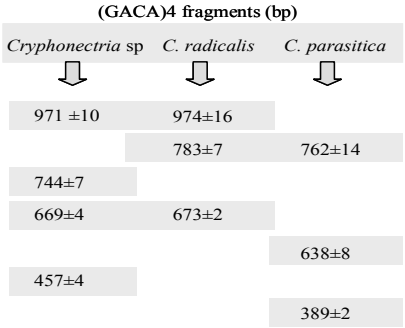

Fig 6 – Size (bp) of main DNA fragments length of $(GACA)_4$ pattern (mean ± standard deviation).

Discussion

The morphological analysis performed in this study made evident that the unknown Portuguese isolates belonged to the new Cryphonectriaceae family, described by Gryzenhout et al. (2006a), and also pointed to their closeness with *C. radicalis* by both PDA culture morphology as well as reddish-purple pigmentation in corn meal medium. However, some of these results, such as the absence of small purple droplets in the Portuguese cultures, did not allow for a conclusion to be drawn as to the identity of the Portuguese isolates being *C. radicalis* (Hoegger et al. 2002).

Subsequently, results obtained through ITS PCR-RFLP methodologies with *Alu* I enzyme enabled to verify the existence of a common profile for the three species complex analysed, being this profile compatible with the one presented by Myburg et al (1999) for *C. parasitica* and distinct from *Endothia gyrosa*. Despite the fact that this molecular method did not discern the three groups in study, it allows to reject the hypothesis that Portuguese *Cryphonectria* sp. isolates were *Endothia gyrosa*.

The results of the phylogenetic analysis performed in this study, supported by those of Myburg et al. (1999, 2004a,b), allowed to conclude that the Portuguese *Cryphonectria* sp. isolates belong to *C. radicalis* phylotype II (Myburg et al. 2004b, Gryzenhout et al. 2006a,b).

Concerning MSP-PCR techniques, the polymorphisms found for each primer, i.e., M13, $(GTG)_5$, and $(GACA)_4$, clearly distinguished three different patterns among the analysed isolates and which correspond to the three species group under study. Among these primers, $(GACA)_4$ produced the most distinctive banding patterns for each group of isolates. A singular and well separated PCR band of 457 bp was found to be specific for *C.radicalis* phylotype II isolates and two specific bands of 389 bp and 638 bp were found to be diagnostic for *C. parasitica*. *C. radicalis* phylotype I could be identified due to the lack of the three diagnostic bands, referred above. These findings indicate that these bands are reliable markers to differentiate easily and in a short time-frame *C. radicalis* phylotype I, *C. radicalis* phylotype II and *C. parasitica*. Moreover, this technique has the potential to differentiate the other species inside the genus *Cryphonectria*, or even inside the family Cryphonectriaceae, since many authors have reported good results with the differentiation of related species using this method (Sampaio et al. 2001, Gadanho et al. 2003, Roque et al. 2006). However, further studies

are needed, with a broader and representative number of isolates from other species, not yet available in Portugal.

Concerning the Portuguese history *of Cryphonectria* spp., maybe this study could provide the start of a remake. In Portugal, *C. parasitica* was first reported on *Castanea crenata* Sieb. & Zucc in 1929 (Camara, 1929), i.e. prior to the first published report in Europe (Biraghi, 1946). But since that identification was based on pycnidia/conidia morphology, compatible with the *C. radicalis* morphological characteristics, the identified fungus could be either *C. parasitica* or *C. radicalis*. This fact could be an explanation to the delay between the 1929 report and the chestnut blight disease first report on *C. sativa* Mill (Abreu 1992).

Based on the presented results and analyzing the phylogenetic results published by Myburg et al. (2004a,b) and Gryzenhout et al. (2006a,b,c) a question arises: could the two phylotypes of *C. radicalis* represent two different species? At present time *C. radicalis* taxonomy remains unclear despite all the knowledge that has been gathered in recent years, mainly because the teleomorphic forms (required for accurate morphological species differentiation) are not commonly found in nature and are not easily produced *in vitro* (Myburg et al. 2004b).

The combination of the morphological and molecular method based on MSP-PCR with $(GACA)_4$ primer, as well as the phylogenetic approach used in this study, could contribute to enlighten the taxonomy at species level of the *C. radicalis* phylotypes. Since species of *Cryphonectria* genus could be either pathogens or saprophytes, correct identification of these isolates is crucial for a complete understanding of their role in chestnut and cork oak decline.

Acknowledgements

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PART IV

FINAL REMARKS AND FUTURE PERSPECTIVES

FINAL REMARKS AND FUTURE PERSPECTIVES

The work herein had the endeavor to describe the current Portuguese situation concerning the epiphytotic chestnut blight disease, assessing the pathogen distribution and determining dispersal biotic and abiotic dispersal factors potentially involved in the broad spread of *C. parasitica* within chestnut stands areas in the country. In addition, an assessment of the biological control potential in Portugal was performed, using an integrated approach, comprising both classic and molecular methodology, intended to identify *C. parasitica* genetic diversity in Portugal, recognize its mode of reproduction and search/introduce hypovirulent forms of the fungus.

Since atypical strains, found to be strongly related with *C. parasitica*, were collected among *C. parasitica* isolates, an additional goal of the present research was to correctly allocate these newly reported isolates to the correct taxon. In fact, the genus *Cryphonectria* groups both pathogenic and saprophytic strains and the correct identification of these isolates is crucial to give initial insights on their role in chestnut ecosystem.

Throughout the present written report, the scientific research developed in the last years was thoroughly summarized and analyzed, hence we will discuss in this chapter only the most relevant results obtained, aiming to promote an holistic approach to these results, capable of an integrative contribution for the scientific knowledge of the etiology of this disease. Although this research also fulfills academic goals, the readers must be aware that this work was the result of an urgent need to respond to numerous governmental and forest service requests for an effort to stop the decay of chestnut stands in the country and to stall the progression of this disease, which at present time poses a serious economic and ecological problem. To delineate the milestones of the present thesis, the premise of a practical application of the results prevailed.

The vast monitoring field work, encompassing most of the distribution of chestnut stands in Portugal (mainland and archipelagos), constituted the basis for which all subsequent work was developed and allowed to determine the current extend of chestnut blight in the country (Chapter II-1).

In the beginning of this decade, a general idea that chestnut blight disease was limited only to localized regions in the Northeastern part of the country persisted and a scarce knowledge of the situation in the rest of the country remained. This situation was linked to the relevance of the Northeastern part of the country, as the largest and greatest important chestnut area in Portugal, which made it the target of multidisciplinary scientific work traditionally linked to this area. However, the present study was pioneer in its geographical range, since an exhaustive survey was made including, not only of areas traditionally known to harbor the disease, but also the rest of the country. It was confirmed in this study that the Northeastern provinces of the country, i.e. Trás-os-Montes e Alto Douro and Beira Interior, are the ones with the highest incidence of the disease, presenting as well the maximum genetic diversity of the pathogen amongst surveyed areas (Chapter II -1).

It was found that the disease reaches, at present time, all chestnut areas of the country with a high incidence in economically relevant chestnut areas but also – and equally important - in areas with a high social and ecological relevance (Chapter II-1). The steady dissemination and progression rate of the disease can compromise the financial stability of populations, which traditionally depend on chestnut products (i.e., nuts, wood, silviculture), and landscape and sociological changes, with critical anthropological and environmental effects, are also forecast. The latter are already occurring in areas such as the Serra de S. Mamede Natural Park, in the North of Alentejo, where areas traditionally devoted to chestnut are experiencing high mortality rates and local populations are progressively less motivated to install new chestnut stands, even if supported by governmental incentives.

Since the disease was first reported in Trás-os-Montes only two decades have passed. The fast dissemination of the disease may be correlated with some aspects of the biology of the fungus. The frequent occurrence of perithecia and the observed mating type ratios indicate that sexual reproduction of *C. parasitica* is common in Portugal (Chapter III-1). Probably, sexual reproduction has played an important role in this rapid epiphytotic disease through the production of wind-borne ascospores, which can disperse over relatively long-distances. It is not common in this species the prevalence of sexual reproduction in regions of recent blight introduction and in which low genetic diversity is observed with one or two vc types prevailing, such as the Portuguese situation. The frequent sexual reproduction in Portugal is most likely the result of the introduction of isolates of both mating types or self-fertile isolates.

It was found that the genetic diversity of *C. parasitica* in Portugal is lower than in other countries where the disease is established earlier; however, it is forecast that this diversity may exponentially increase due to sexual recombination.

The present low diversity of *C. parasitica*, associated with the existence of hypovirulent strains of the fungus, may contribute to the natural control of the disease in Portugal. The dsDNA hypovirus of *C. parasitica* are transmitted preferentially between vegetative self compatible individuals, hence, the fact that hypovirulent strains found in Portugal belongs to the dominant vc type in country will promote natural dissemination of hypoviruelnce. However, if low vc type diversity will favor hypovirus invasion of *C. parasitica* populations, conversely, the frequent sexual reproduction observed could pose a major obstacle to the spread of the hypovirus due to the potential increase of vc type diversity through sexual recombination and the production of hypovirus-free ascospores.

Although the low incidence of hypovirulence in Portugal could be attributed to a recent introduction of the hypovirus, the facts that only a single hypovirulent isolate was obtained could be related with scarce knowledge of specific signs linked with hypovirulence (e.g. observation of healed cankers) coupled with the fact that virulent and hypovirulent strains may co-exist and, therefore, single isolations become a difficult task. The hypovirulent strains, which show a slower growth rate in culture, can be rapidly overcome by the fast growth of the virulent strains. Cases were detected during this research in which hypovirulence was hypothesized due to evidence of scar tissue in cankers, although cultures with typical hypovirulent morphology could not be obtained from these samples.

As a consequence, the incidence and frequency of hypovirulence in Portugal may in fact be higher than the one hereby reported.

Concerning the abiotic factors analysed in the study case described in chapter II-2, the majority of the cankers in the trunk and branches were detected in Southern-exposed locations and, in general, a low relationship was found between the degree of canker incidence and environmental factors. These facts reinforce the general idea that cultural practices may be more important for the incidence and dissemination of *C. parasitica* than other factors. The higher frequency of cankers in mature trees could be explained by the higher levels of human intervention on these productive trees, which are subjected to frequent pruning activities and incorrect sanitary operations. Furthermore, the increasing value of the chestnut fruit resulted in intensified grove

exploitation. Although other agents may act as dispersal vectors of *C. parasitica*, such as insects and birds, pruning is probably the most important factor for the local spread of the blight. In fact, intensive pruning (aiming to induce fruit production) is a recurring management practice in local chestnut groves, using hatchets and saws which are seldom disinfected during cutting actions, and therefore can disseminate chestnut blight throughout the groves. Future research should focus on the identification and influence of biotic factors, such as insects or birds, in the natural dispersal and characterization of the disease.

Overall, our results suggest that the implementation of good management practices in Portugal may be of fundamental importance to prevent further dissemination of chestnut blight, reduce stress on chestnut trees and thus enhance the establishment of natural hypovirulence.

Natural dissemination of hypovirulence, in opposition to artificially introduced strains, has been found to be more successful in recovering areas affected by the blight. Nevertheless, there are some cases of successful biocontrol with introduced hypovirulence. At the individual canker level, therapeutic treatment with hypovirulence is highly successful, and it is relevant for intensively managed, high-valued orchards or plantations.

Due to the fact that some success has been achieved with hypovirulence, and since at present time efficient biological control alternatives for the treatment of this disease are not known, biologic control must be tested in our country. The results and thorough analyses of the biology, genetic and geographic distribution of the disease in Portugal obtained in this research have gather the suitable conditions and knowledge for a secure implementation of a biological control management with controlled and restricted supervised methodologies.

The implementation of biological control should not increase the potential for recombination in the local population and therefore the hypovirulent strains should always belong to a vc type existing in the area, preferentially, the prevailing natural occurring vc type in the region, since efficacious transmission of the hypovirus will be enhanced. In addition, the introduction of hypovirulent strains in restricted areas should only be justified if asexual reproduction prevails, since the hypovirus is only transmitted through this type of reproduction. For the safe utilization of this technique in an ecological and environmental large scale, a robust scientific know-how is required,

which is at present time economically unviable in the context of the Portuguese economy.

Concerning the last topic discussed in this dissertation (Chapter III-2), the results achieved are of extreme importance and may considerably help to clarify doubts about *C. parasitica* and/or related species, which have remained unsolved for a long period of time. The first Portuguese report of the fungus *Endothiella gyrosa* was made by Professor Câmara in *Quercus suber*, which also described *Cryphonectria parasitica* in *Castanea sativa*, as referred in Chapter III-2, but a question mark ("?") was included by him after the fungus name in the publication. Subsequently, another phytopathologist, Oliveira, compared the Portuguese *Endothiella gyrosa* with specimens from an American *Endothia* collection and further referred that morphologic characteristics of the Portuguese fungi were not sufficient for a conclusive identification. Until now the *Endothiella* fungus was referred in Portugal as the causal agent of "ferrugem alaranjada" ("orange rust") disease on cork oak.

The analysis performed on the *Endothiella* specimens obtained from these scientists, and kept in herbarium collection, were not conclusive and did not allow to clearly discern the taxonomical position of these strains, since the isolates in herbarium were only the anamorph (i.e., imperfect form) of the fungus.

Several questions arose or were reopened during the development of the present work which require further analysis and should constitute important lines of research in the nearest future. Could the parasitic fungus found in cork oak and referred to as *Endothiella gyrosa* be the anamorphic form of *Cryphonectria parasitica* or *Endothia gyrosa*? As previously referred (Chapter I and chapter III-2), these two species belong to the same family of parasitic fungi, sharing the same host and are difficult to distinguish in their anamorphic forms.

The fact that the same author, in the same year (1929), reported for the first time *C. parasitica* and *Endothiella gyrosa*, and also the different sizes of conidia referred, lead us to hypothesize that these were effectively different fungi. Both identifications were based on the anamorphic form and, mainly in *C. parasitica* reference, the description is fully compatible with the one of the saprophytic species *Cryphonectria radicalis*.

The fact that the culture designated by Oliveira as *Endothiella gyrosa* has been recently re-classified, using phylogenetic analysis, as *C. radicalis* (phylotype II) could be the key to clarify these taxonomic issues, although two main questions still remain:

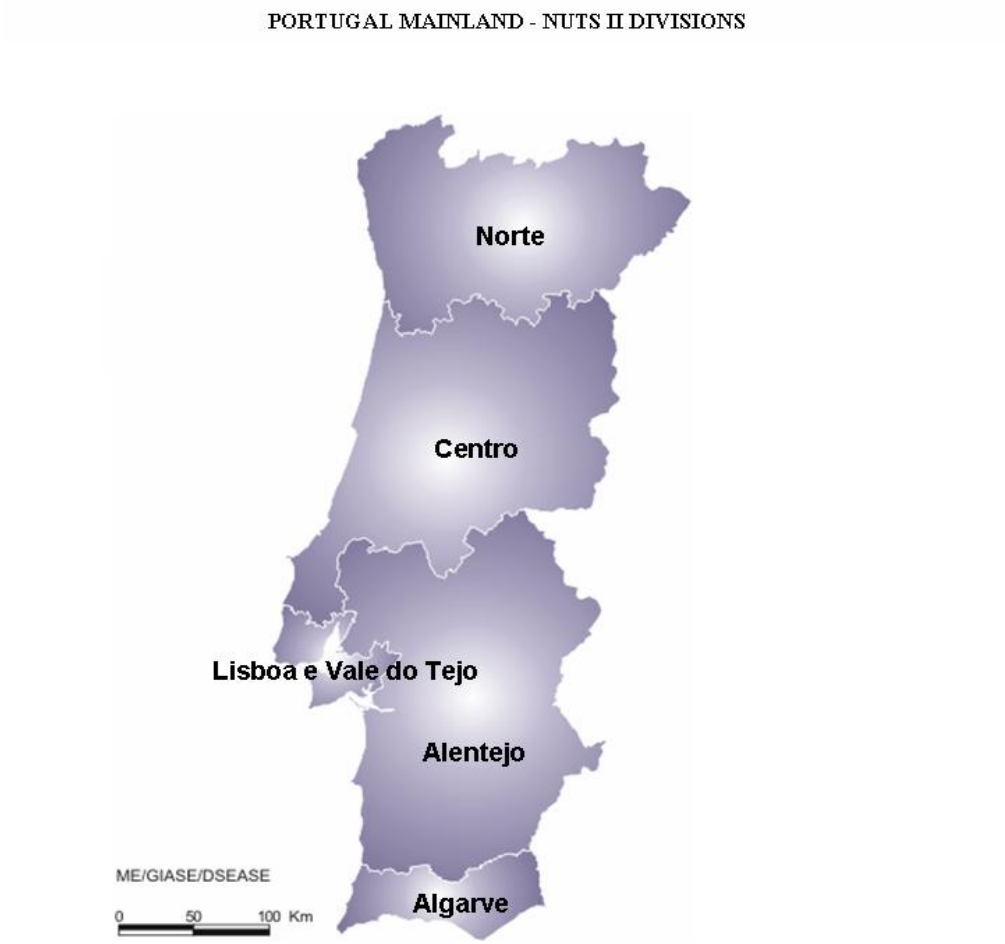
Could *C. radicalis* have a parasitic behavior in the context of the Portuguese cork oak ecosystem? Or in alternative could be even a new species of *Cryphonectria*?

We believe that the results achieved and presented in this thesis contribute to the understanding of the etiology of the epiphytotic chestnut blight disease in Portugal, in particular, and to the improvement of the scientific knowledge in landscape pathology in general.

Although the current situation that research at the ex-Estação Florestal Nacional is facing is not promising, we hope the current report will be not only a contribution for forest science in Portugal but also that it will raise awareness and safeguard of the ecological patrimony, and recognized ancestral heritage, that forest stands own in Portugal. These stands require protection and investment, following the current trend in the rest of the world, after the general recognition of the critical importance and interaction of forest ecosystems in all aspects of human life.

ANNEXES

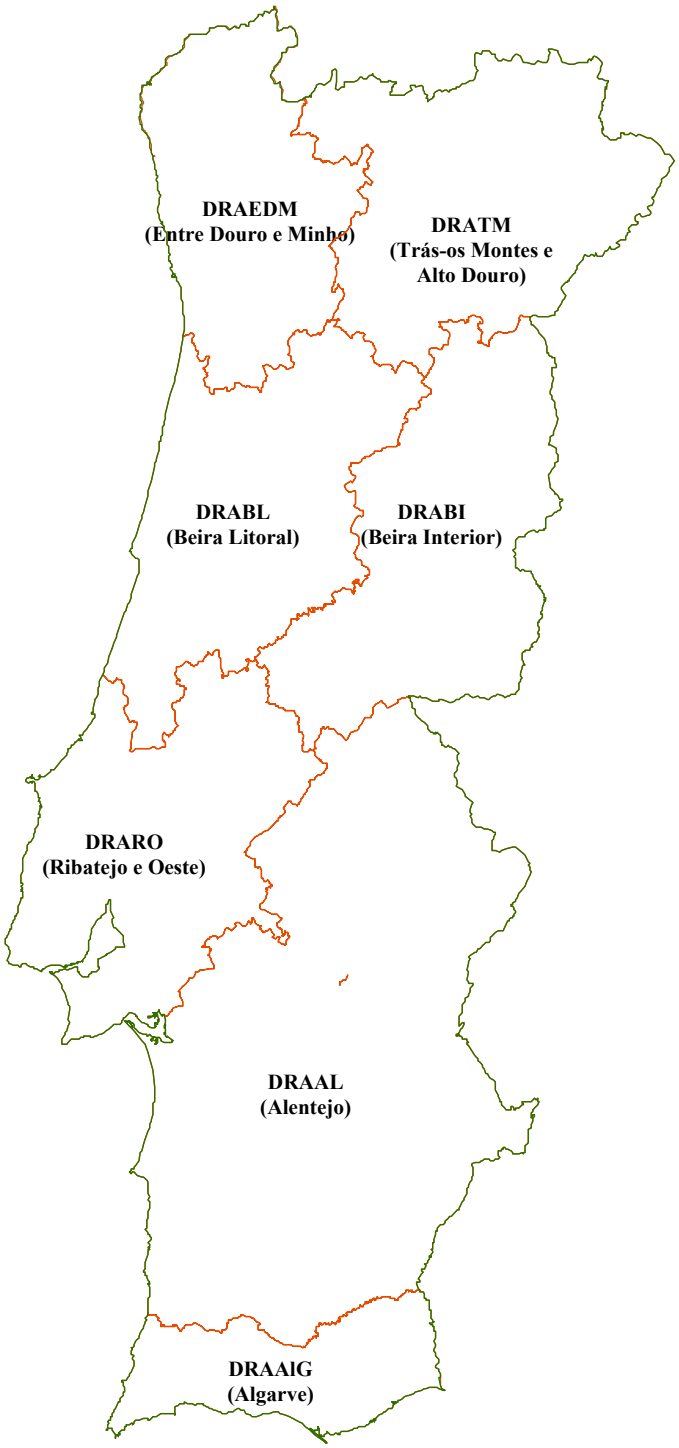
ANNEX I



From: http://www.giase.min-edu.pt/BasesTerritoriais/continentecomnuts2.htm

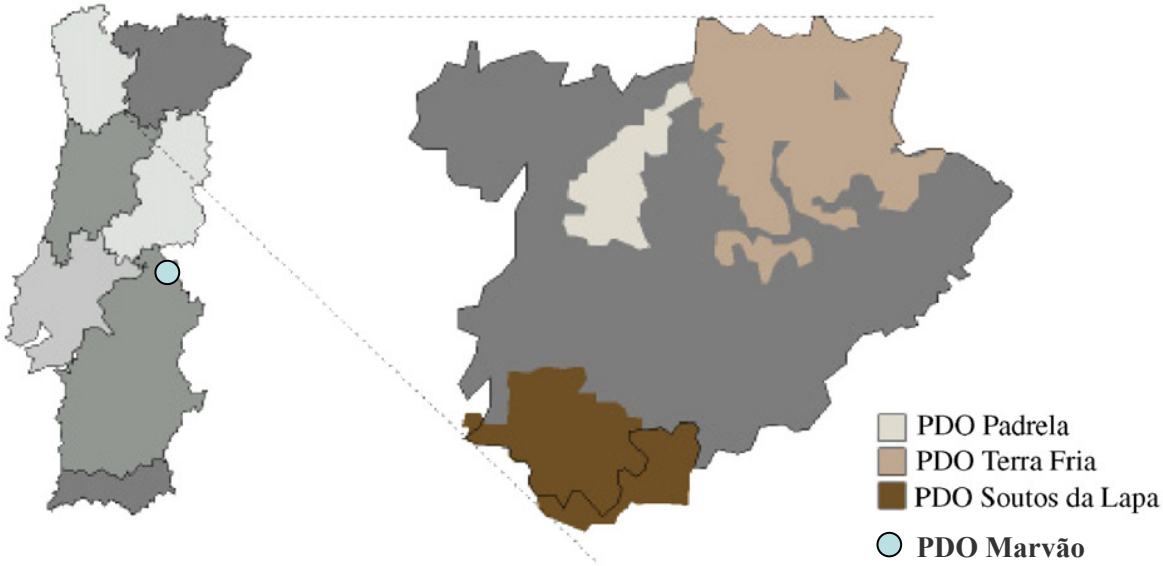
ANNEX II

Agricultural Services Areas in Portugal Mainland



ANNEX III

Chestnut Fruit - Protected Denomination of Origin Areas



Adapted from: Borges, O.P.; Soeiro Carvalho, J.; Reis Correia, P.; Paula Silva, A., 2007. Lipid and fatty acid profiles of Castanea sativa Mill. Chestnuts of 17 native Portuguese cultivars. Journal of Food Composition and Analysis. *Food Composition and Analysis*, 20 (2):80-89.

ANNEX IV

GRYZENHOUT *et al.* (2006c) provided the following key to facilitate the distinction between different diaporthalean genera with orange stromatic tissue:

1a. Conidiomata pyriform to clavate; ascostromata with reduced stromatic tissue..... 2
1b. Conidiomata pulvinate; ascostromata well-developed..... 4
2a. Conidiomata black; orange ascostroma with black perithecial necks..... ***Chrysoporthe***
2b. Conidiomata orange..... 3
3a. Conidiomata rostrate with tapered necks; orange stroma with orange perithecial necks.....***Rostraureum***
3b. Conidiomata pyriform or rostrate or globose with more cylindrical necks; teleomorph unknown.....***Ursicollum***
4a. Ascospores septate..... 5
4b. Ascospores aseptate..... 6
5a. Ascostromata large, well-developed, semi-immersed; paraphyses absent in conidial locules.....***Cryphonectria***
5b. Ascostromata small to medium size, usually superficial; conidial locules containing paraphyses..... ***Microthia***
6a. Ascostromata large, well-developed, superficial..... ***Endothia***
6b. Ascostromata small to medium size, semi-immersed..... ***Holocryphia***