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Clonal population structure and introductions of the chestnut blight fungus, *Cryphonectria parasitica*, in Asturias, northern Spain

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Abstract To understand the history of introductions of the chestnut blight fungus, *Cryphonectria parasitica*, in the Principality of Asturias in northern Spain, we conducted an extensive survey of chestnut blight and collected *C. parasitica* from 216 sites. All 778 isolates were assayed for vegetative compatibility (vc) type, whereas a subsample of 301 isolates was assayed for mating type, and 189 isolates were genotyped at 16 microsatellite markers. We found low diversity for all markers. Nearly all isolates (95%) were compatible with vc type EU-1 and had the same microsatellite multilocus haplotype, or differed from the most common type by mutation at one locus. Approximately 5% of the isolates were vegetatively compatible with EU-13 and only two isolates (< 1%) were compatible with EU-3; five different microsatellite haplotypes were found among isolates in these latter two vc types. The overall mating-type ratio was 218 *MAT-1*: 81 *MAT-2*, with both mating types represented in each of the three vc types. Microsatellite haplotypes based on ten markers used in France showed that most isolates in Asturias were either identical to or only one marker different from one of the seven most common genotypes in France, RE103. Based on these ten markers alone, the population of *C. parasitica* in Asturias, would appear to have been founded by a single genotype from the C1 lineage (to which RE103 belongs) found in eastern France and northern Italy. However, additional genotyping by vc types suggests the introduction of multiple genotypes, with different vc types. The exact source for introduction into Asturias cannot be determined without additional genotyping of isolates from other locations. Regardless of their origin, the low diversity of vc types makes this population ideal for deploying hypovirulence because there will be few barriers for virus transmission between individuals.

Keywords Biological invasion · Forest pathogen · Founder effect · Mating type · Microsatellite genotyping · Vegetative compatibility type

Abbreviations

CHV-1	*Cryphonectria hypovirus 1*
vc type	Vegetative compatibility type
vic	Vegetative incompatibility gene

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Introduction

Plant pathogens have been notorious invasive organisms that have caused severe economic losses to

crops, ornamental plants and forests. Despite the significance of these biological invasions, relatively little is known about how most fungal pathogens are introduced or how many times and when they are introduced (Desprez-Loustau et al. 2007). Understanding these processes is important for predicting, preventing and responding to additional introductions.

One of the most infamous introduced plant pathogens is the ascomycete fungus *Cryphonectria parasitica*, which causes chestnut blight on species in the genus *Castanea*. This fungus was introduced into North America and Europe from east Asia (Milgroom et al. 1996) and subsequently resulted in the destruction of chestnut forests on both continents (Anagnostakis 1987). *C. parasitica* was first documented in Europe in northern Italy, near Genoa in 1938 (Biraghi 1946), and spread quickly throughout Italy and into southern France and Switzerland (Heiniger and Rigling 1994). It later spread eastwards into the Balkans, and westwards to Spain and Portugal (Robin and Heiniger 2001). In southeastern Europe, a single clone of *C. parasitica*, characterized by vegetative compatibility (vc) types, neutral molecular markers and mating type, spread across southern Italy and the southern Balkans (Milgroom et al. 2008). To the west, a recent study (Dutech et al. 2010) of the population structure of *C. parasitica* in France using microsatellite markers found three separate gene pools (or clonal lineages) that do not recombine, despite the fact that both mating types are present. These authors concluded that *C. parasitica* had been introduced at least three times. Founders of one of the clonal lineages (C1) probably migrated from northern Italy, whereas the other two (C2 and C3) represent separate introductions that are unrelated to the one in Italy. Lineage C1 is most common in southeastern France, whereas C2 and C3 are most common in southwestern France, particularly near the Pyrenees. The actual dates, sources and locations of introductions are not known, and there is some uncertainty as to whether they may have occurred as early as 1920 (Dutech et al. 2010).

Less is known about the introductions of *C. parasitica* into Spain and Portugal. Historical records indicate that chestnut blight was first detected in northern Spain in 1947, near Bilbao (Elorrieta 1949). Chestnut blight has since been reported from most of the chestnut-growing areas of Spain and Portugal. Most populations in Spain and Portugal are dominated by a single vc type, with a few less common or rare vc types present. Interestingly, different vc types dominate in different locations. For example, vc type EU-66 is dominant in Navarra (Robin et al. 2009) and Galicia (Montenegro et al. 2008), whereas EU-2 is dominant in Catalonia (Trestic et al. 2001), EU-1 in León (Montenegro et al. 2008) and EU-11 in Trás-os-Montes in northern Portugal (Bragança et al. 2007). Robin et al. (2009) speculated that dominant vc types might represent clonal lineages and separate introductions. Mating-type frequencies varied also. Both mating types occurred at approximately equal frequencies in populations in France (Robin et al. 2009) and Portugal (Bragança et al. 2007), but *MAT-1* was dominant in Galicia (Montenegro et al. 2008) and *MAT-2* was dominant in Navarra (Robin et al. 2009). The presence of both mating types in many populations indicates a potential for sexual reproduction, although their presence does not necessarily indicate that recombination has occurred (Milgroom et al. 2008; Dutech et al. 2010).

The markedly different distributions of vc types among localities across northern Spain and Portugal suggests that each area may have been founded independently. Although surveys of vc types provide a good first approximation for assessing diversity (Breuillin et al. 2006; Milgroom et al. 2008) and the potential for biological control with hypovirulence (Milgroom and Cortesi 2004),vc type is not a refined enough marker to unravel the history of introduction or the spread of *C. parasitica*. Genotyping with neutral molecular markers would allow us to address questions concerning the population structure and history of introductions, as has been done in other parts of Europe (Milgroom et al. 2008; Dutech et al. 2010). To our knowledge, molecular genotyping has not been done previously on *C. parasitica* populations in Spain or Portugal.

In the Principality of Asturias, an autonomous community in northern Spain (Fig. 1a), there are approximately 60,000 ha of chestnut. Chestnut blight was first reported in Asturias in 1982 and by 2000 had spread throughout much of the area (Valdezate et al. 2001) (Fig. 1a). Because of the economic and social significance of chestnut and the seriousness of chestnut blight in Asturias, our objectives were to determine the current distribution of the disease and to understand the introduction and invasion of *C. parasitica*. Our specific objectives were to assess the genetic diversity of *C. parasitica* with respect to vc

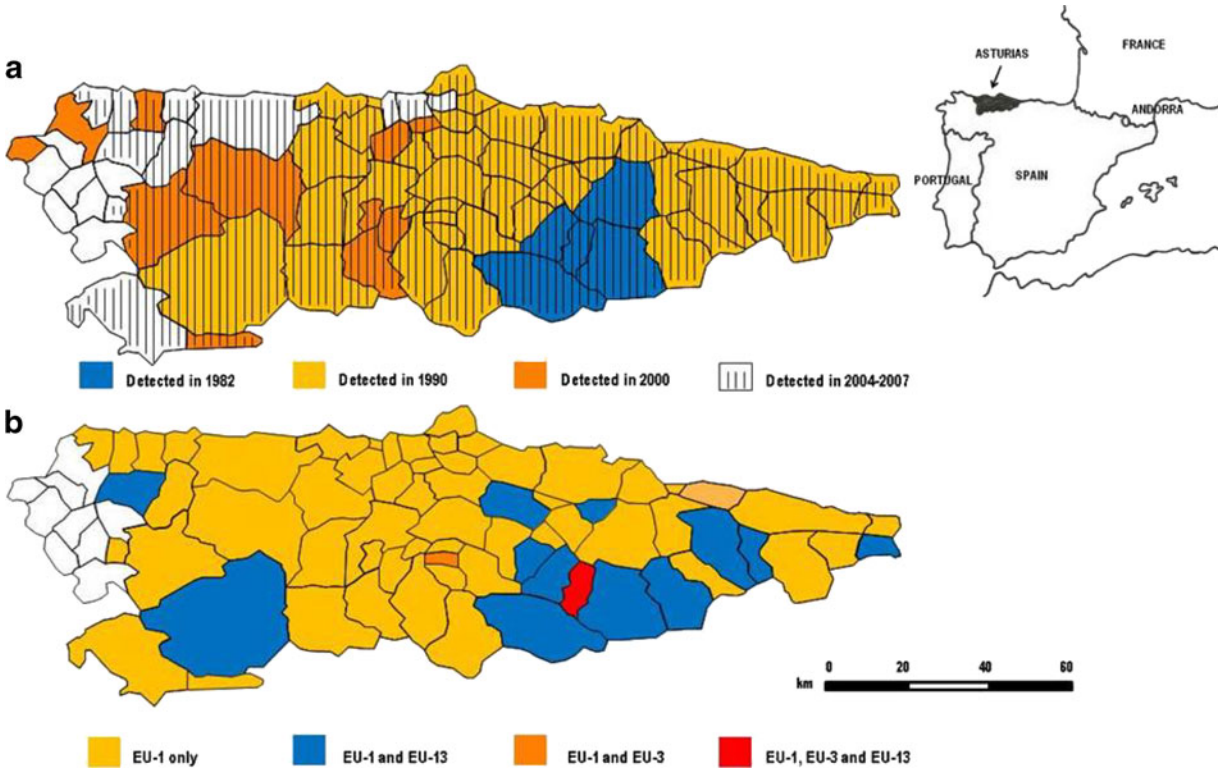

Fig. 1 Map of the Principality of Asturias, an autonomous community in northern Spain, showing **a**) historical distributions of chestnut blight, caused by *Cryphonectria parasitica*, and **b**) distribution of vegetative compatibility (vc) types in 2005–2007. The distribution of blight before 2005 was reported by Valdezate et al. (2001)

types and distribution of mating types, and to test the hypothesis that populations are clonal by genotyping with microsatellite markers. Our last objective was to compare genotypes of *C. parasitica* from Asturias to those reported from southern France to see if the pathogen could have been introduced into northern Spain from France.

Materials and methods

Sampling *Cryphonectria parasitica*

We looked for the presence of chestnut blight in all 78 councils in the Principality of Asturias in 2005–2007 by visiting several sampling sites within each council where chestnut trees were found. Sites varied considerably but can be described, from approximately the most common to the least common, as: forest stands for wood production, trees associated with grasslands, isolated trees by roadsides, tree rows between fields, orchards for nut production, or isolated old trees in city parks. In each sampling site, we examined trees for symptoms of chestnut blight. Using a sharp tool disinfected with a 10% bleach solution (ca. 0.5% sodium hypochlorite), we cut a piece of bark (approximately 5×5 cm) from the margin of each canker sampled. We sampled one canker per tree to avoid sampling clones (Milgroom and Lipari 1995). Within a site, we sampled from trees separated by at least 10 m to avoid clones (Robin et al. 2000). We collected a maximum of ten samples per site to sample evenly throughout Asturias.

Bark samples were kept at room temperature for 24–48 h until they were processed in the lab. Using a sterile cork-borer (5 mm diameter) we cut two bark discs per sample to increase the chances of isolating *C. parasitica*. Discs were surface-disinfected in 70% ethanol for 15 s, then rinsed in sterile water and placed upright in Petri plates containing potato dextrose agar (PDA) amended with 2 ml/l lactic acid after autoclaving. Plates were maintained in the dark at 25°C for 3–5 days. Mycelium from one bark disc was transferred to a new PDA plate and incubated in

the same conditions. We transferred mycelia from both discs in rare instances (< 1%) where isolates differed in colour, e.g., white and orange.

From each isolate, we made single-conidial or hyphal-fragment cultures by serial dilution. From dilution plates with one or two well separated macroscopic colonies, we transferred one isolate to PDAmb (PDA enriched with 0.1 g/l methionine and 0.001 g/l biotin) (Anagnostakis 1984). Cultures were grown in the dark at 25°C for 5 days, and then stored at 4°C.

In addition to isolates we collected in 2005–2007, we also obtained 136 isolates from preliminary studies of chestnut blight in Asturias (Table 1). We obtained 25 isolates from the Laboratorio de Sanidad Vegetal del Principado de Asturias, which were collected in nine councils in 1999–2000 (Valdezate et al. 2001); and 111 isolates from the Area Forestal del SERIDA, which were collected from a single council (Aller) in 2002–2003.

DNA extraction

Mycelia of each isolate were transferred to PDA plates overlaid with sterile cellophane and grown in the dark at 25°C for 5 days. Using a sterile toothpick we harvested an area of mycelium approximately 3× 3 cm and placed it in a 2 ml microcentrifuge tube. We added 350 µl of lysis buffer (400 mM Tris–HCl, pH: 8.0; 60 mM EDTA, pH: 8.0; 150 mM NaCl; 1% SDS; 50 µl/ml proteinase k (20 mg/ml)) and incubated at 30 min at room temperature. We added a sterile glass ball (Ø=3 mm) and 100 µl of acid-washed glass beads (≤106 µm, SIGMA-Aldrich Inc., USA) and shook for 15 min in a Restsch MM2000 shaker (Restsch, Germany) at maximum power followed by 15 min on ice. This shaking procedure was done three times, after which we added 150 µl 3M sodium acetate, incubated for 10 min at −20°C before centrifuging at 14,000 rpm for 2 min. The supernatant was transferred to a new tube and we added 450 µl phenol and 450 µl chloroform-isoamyl alcohol (24:1). Tubes were shaken, then centrifuged at 14,000 rpm for 5 min and the supernatant was transferred to a new tube. This step was repeated as many times as necessary to get a clean supernatant, doing the last step with only chloroform-isoamyl alcohol (24:1). The supernatant were transferred to a new tube and we added 5 µl RNase (5 mg/ml) (Roche Diagnostics S.L., Spain) and incubated at 37°C for 15–30 min. We added 125 µl 7.5M ammonium acetate and 800 µl ethanol (−20°C) and stored overnight at −20°C. DNA was precipitated by centrifuging at 14,000 rpm for 15 min. After discarding the supernatant, we added 100 µl 70% ethanol (−20°C), centrifuged at 14000 rpm for 5 min and discarded supernatant and dried the pellet at 37°C 15–30 min. We added 20 µl TE to resuspend the DNA and stored it at −20°C.

Vegetative compatibility typing

Vegetative incompatibility was assayed by growing pairs of isolates together and looking for a barrage (Cortesi et al. 1996). We transferred conidia of each isolate using a sterile toothpick (Cortesi et al. 1998) to PDA with bromocresol green (50 mg/l) (Powell 1995). Plates were incubated at 25°C for 7 days in the dark, followed by 7 days in the light (16 h light/ 8 h dark) at which time barrages could be observed between colonies if they were incompatible.

We used a hierarchical strategy to determine the number of vc types (Cortesi et al. 1996). We first paired all isolates from the same site to group compatible isolates of the same vc type. One isolate was randomly chosen to represent each vc type in that site. Then we paired the representative isolates of all vc types among sites within a council and then all vc types among councils to determine the total number of vc types in Asturias. Representative isolates from each vc type were paired with 64 EU tester strains of known vegetative incompatibility (*vic*) genotypes (Cortesi and Milgroom 1998). Each pairing was repeated a minimum four times.

Vc type diversity was estimated as described by Grünwald et al. (2003) and applied to *C. parasitica* by Sotirovski et al. (2004). Richness (*S*) was calculated as the number of vc types found in each population (council). The maximum number of genotypes is $g_{max}=2^k$, where k is the number of polymorphic *vic* loci in a population (Cortesi and Milgroom 1998). The Shannon index of diversity (H') was calculated as $H' = -\sum_{i=1}^{S} p_i \ln p_i$, where p_i is the frequency of the i^{th} vc type in each population (council). Genotypic diversity ($\hat{G}$) was calculated as $\widehat{G} = 1/\sum_{i=1}^{S} p_i^2$. Evenness ($E_5$) was calculated as $E_5 = (\widehat{G} - 1)/(e^{H'} - 1)$.

Table 1 Vegetative compatibility type and mating type of isolates of *Cryphonectria parasitica* in Asturias

Council	No. sampling sites	*N*	vc type			Mating type		
			EU-1	EU-3	EU-13	*MAT-1*	*MAT-2*	*MAT-1/2*
Allande	3	9	9			4		
Aller	–	111 (111)[a]	106		5	4		1
Amieva	3	6 (6)	6				5	
Avilés	1	4	4			4		
Belmonte	4	13 (3)	13			3	2	
Bimenes	2	12	12			3	2	
Boal	5	10	8		2	2	3	
Cabrales	2	4	4			4		
Cabranes	2	9	8		1	4	1	
Candamo	2	6 (6)	6			4		
Cangas de Onís	3	19[b]	18		1	3	3	
Cangas del Narcea	4	18[b]	15		3	6	1	
Caravia	2	4	4			3	1	
Carreño	4	13 (2)	13			4	1	
Caso	3	17 (2)	13		4	5	1	
Castrillón	2	5	5			5		
Castropol	2	0[c]						
Coaña	2	3 (2)	3			3		
Colunga	1	4	4			1	3	
Corvera	1	2 (1)	2			2		
Cudillero	2	7	7			2	3	
Degaña	3	2	2			1	1	
El Franco	1	2	2			2		
Gijón	5	14[b]	14			5		
Gozón	4	10	10			4	1	
Grado	4	17[b] (1)	17			4	1	
Grandas de Salime	3	0						
Ibias	5	4	4			3	1	
Illano	2	0						
Illas	2	2 (2)	2			2		
Langreo	3	9	9			4	1	
Las Regueras	2	7	7			4	1	
Laviana	2	12	10		2	2	2	
Lena	7	35	35			5		
Llanera	3	2	2			1	1	
Llanes	4	16[b]	16			3	2	
Mieres	4	22[b]	22			3	2	
Morcín	5	17	16	1		4	1	
Muros del Nalón	2	8	8			4	1	
Nava	2	7[b]	7			3	1	
Navia	1	2	2			2		
Noreña	1	4[b]	4			4		
Onís	2	9	6		3	3	2	
Oviedo	2	11	11			3	2	
Parres	4	16	16			2	2	

Table 1 (continued)

Council	No. sampling sites	*N*	vc type			Mating type		
			EU-1	EU-3	EU-13	*MAT-1*	*MAT-2*	*MAT-1/2*
Peñamellera Alta	4	19	19			4	1	
Peñamellera Baja	3	11[b]	8		3	2	1	1
Pesoz	2	2	2			2		
Piloña	5	17[b]	17			4	1	
Ponga	5	24[b]	16		8	3	2	
Pravia	2	8	8			3	2	
Proaza	2	10	10			5		
Quirós	4	7	7			5		
Ribadedeva	1	5[b]	5			2	3	
Ribadesella	1	4	4				3	
Ribera de Arriba	3	18	18			2	3	
Riosa	3	10	10			3	2	
Salas	3	6	6			3	2	
San Martín de Oscos	2	0						
San Martín del Rey Aurelio	4	14[b]	11		3	5		
San Tirso de Abres	3	0						
Santa Eulalia de Oscos	2	0						
Santo Adriano	1	4	4			3	1	
Sariego	2	4[b]	4			4		
Siero	5	20[b]	19		1	3	2	
Sobrescobio	4	17	12	1	4	5		
Somiedo	3	13	13			5		
Soto del Barco	2	7	7			3	2	
Tapia de Casariego	2	1	1			1		
Taramundi	2	0						
Teverga	3	13	13			5		
Tineo	2	4	4			4		
Valdés	4	5	5				5	
Vegadeo	2	0						
Villanueva de Oscos	3	0						
Villaviciosa	6	24	24			3	2	
Villayón	1	1	1			1		
Yernes y Tameza	2	6[b]	6			4	1	
Total	216	778 (136)	736	2	40	218	81	2

[a] Numbers in parentheses indicate isolates collected previously by Área Forestal del SERIDA and the Laboratorio de Sanidad Vegetal del Principado de Asturias

[b] Councils in which some isolates were white, indicating the possibility that they are infected with CHV-1

[c] No isolates of *C. parasitica* were successfully obtained

Mating-type assay

We assayed 301 isolates for mating type by PCR as described McGuire et al. (2004) by sampling a small number of isolates from each council. PCR was done using primers M1-GS1n and M1-GS3-rev for *MAT-1*, and M2-GS3 and gs1-d-1 for *MAT-2*. We used a chi-square goodness of fit test to test for 1:1 ratios, which would be expected in randomly mating populations.

Microsatellite genotyping

We genotyped 189 isolates with 16 microsatellite markers as described by Kubisiak et al. (2007). Using the sampling strategy of Milgroom et al. (2008), we sampled isolates randomly from the dominant vc type and mating type in each council, and genotyped all isolates in rare vc types and mating type (see Results below). The 16 markers included: *I07-650* (Davis et al. 2005), *CPE1, CPE3, CPE4, CPE5, CPE8, CPG3, CPG4* (Breuillin et al. 2006), *CPG6, CPG14, CPG7, CpSI006, CpSI009, CpSI032, CpSI054* and *CpSI072* (Kubisiak et al. 2007). The first 10 of these markers were used by Dutech et al. (2008; 2010) to genotype *C. parasitica* in southwestern France.

Following methods of Kubisiak et al. (2007), we added an M13-specific sequence (5′-CACGA CGTTGTAAAACGAC-3′) to the 5′-end of each forward primer (Schuelke 2000) to detect microsatellite fragments. Briefly, PCR was performed with primer pairs specific to each microsatellite locus, plus a complementary M13-specific primer that was 5′-labeled with a fluorescent dye (FAM, VIC, PET, or NED; Applied Biosystems, Carlsbad, CA, USA). PCR products from five or six reactions were pooled for multiplex fragment analysis. Pooling was done with PCR products with different fluorescent dyes, or with the same dye but markedly different allele sizes. Fragment analyses were conducted by the Cornell University Life Sciences Core Laboratories Center using an Applied Biosystems 3730xl DNA Analyzer. Allele sizes were analyzed using the GeneMapper Software v3.0 (Applied Biosystems). To compare allele sizes from our fragment analysis with previous studies using the same markers (Dutech et al. 2008; Dutech et al. 2010), we genotyped DNA from seven isolates from France, generously provided by C. Robin and C. Dutech (INRA, Bordeaux). Altogether, these seven isolates carried 39 alleles at the same ten loci as used in France.

To show relationships among multilocus haplotypes we constructed a minimum-spanning haplotype network (Fitch 1997; Posada and Crandall 2001), as applied previously to *C. parasitica* (McGuire et al. 2005; Milgroom et al. 2008). The network was constructed manually to connect haplotypes with the minimum number of mutational steps.

Results

Distribution of chestnut blight in Asturias

Until 2000, chestnut blight had been reported in 60 of the 78 councils in Asturias (Valdezate et al. 2001), with the earliest reports in the southeastern councils (Fig. 1a). By 2007, we detected chestnut blight in 69 councils, representing more than 95% of the area where chestnuts occur. From these 69 councils, we obtained a total of 618 isolates in 2005–2007. From 24 of the bark samples, we obtained two isolates because colonies from dilution plating were different colors. Together with 136 isolates from previous sources, we analyzed a total of 778 isolates.

Vc type diversity

We found three vc types among 778 isolates, all of which were compatible with known vc types: EU-1, EU-13 and EU-3 (Cortesi and Milgroom 1998). EU-1 was the dominant vc type, with 736 of 778 isolates (94.6%); 40 isolates were compatible with EU-13 (5%) and two were compatible with EU-3 (< 1%). EU-1 was found in all the 69 councils where chestnut blight was found, and was the only vc type in 55 councils (Table 1). EU-13 was found in 13 councils, and EU-3 was found in two (Fig. 1b). All three vc types were found together in only one council (Tables 1 and 2). All councils with more than one vc type, with the exceptions of Boal and Cangas del Narcea, are located in the central-south-east area, where blight has been present the longest. The diversity of vc types was low in all councils and in Asturias overall, regardless of which diversity index is considered (Table 2).

Based on the *vic* genotypes of EU-1, EU-3 and EU-13 (Cortesi and Milgroom 1998), the maximum number of vc types (g_{max}) was calculated within each council assuming recombination between the vc types present (Table 2). In the 13 councils where EU-1 and EU-13 occurred, $g_{max}=16$ because these two vc types differ at four *vic* loci. In the one council where EU-1 occurred with EU-3 (Morcín), $g_{max}=2$. EU-3 differs from EU-1 at one *vic* locus and from EU-13 at three *vic* loci and could, in theory, be a recombinant vc type between the two.

Table 2 Diversity of vegetative compatibility types in councils with more than one vc type

Council	*N*	vc type			*S*	g_{max}	*H′*	$\hat{G}$	E_5
		EU-1	EU-3	EU-13					
Aller	111	106	–	5	2	16	0.18	1.09	0.47
Boal	10	8	–	2	2	16	0.50	1.47	0.72
Cabranes	9	8	–	1	2	16	0.35	1.25	0.59
Cangas de Onís	19	18	–	1	2	16	0.21	1.11	0.48
Cangas del Narcea	18	15	–	3	2	16	0.45	1.38	0.68
Caso	17	13	–	4	2	16	0.54	1.56	0.77
Laviana	12	10	–	2	2	16	0.45	1.38	0.68
Morcín	17	16	1	–	2	2	0.22	1.12	0.49
Onís	9	6	–	3	2	16	0.64	1.80	0.89
Peñamellera Baja	11	8	–	3	2	16	0.58	1.66	0.82
Ponga	24	16	–	8	2	16	0.64	1.80	0.89
San Martín del Rey Aurelio	14	11	–	3	2	16	0.52	1.51	0.75
Siero	20	19	–	1	2	16	0.20	1.10	0.48
Sobrescobio	17	12	1	4	3	16	0.75	1.79	0.71

S = observed richness; g_{max} = maximum number of genotypes possible based on *vic* genotypes; *H′* = Shannon index of diversity; $\hat{G}$ = genotypic diversity; E_5 = evenness

Mating type

We found an overall *MAT-1*:*MAT-2* ratio of 218:81 (0.72:0.27) among 301 isolates, which is significantly different from 1:1 (χ^2=62.77; df=1; P<0.001). Both mating types were found in all three vc types at ratios of 206:77, 11: 3, and 1:1 for EU-1, EU-13 and EU-3, respectively. Both mating-type idiomorphs were amplified from two EU-1 isolates, and are considered heterokaryons (McGuire et al. 2004). The two mating types were widely distributed among the councils, occurring together in 42 of the 69 councils (Table 1). *MAT-1* was found alone in 24 councils and *MAT-2* alone in three councils. The council Aller was considered to have both idiomorphs because one isolate was a heterokaryon.

Microsatellite genotypes

All 16 microsatellite markers amplified successfully, with the exception of locus CPE4 in four isolates despite repeated attempts. Seven microsatellite loci (*CPG6, CPE4, CpSI006, CpSI0032, CpSI054, CPE5, CPG7*) were monomorphic. Nine loci were polymorphic, five with two alleles (*CPE1, CPG3, I07-650, CpSI009, CPE3*), three with three alleles (*CpSI072, CPE8, CPG4*) and one with four alleles (*CPG4*) (Table 3). Genotyping was repeated for loci in all isolates showing polymorphism relative to the most common haplotype.

Allele sizes for nine of the ten loci were 17 to 21 bp larger than sizes reported previously for the same isolates from France (Table 3). Most of this difference is because we added a 19-bp M13 adapter to primers to enable fluorescent fragment detection (Schuelke 2000). Additional variation of ±1–2 bp is most likely the result of using different methods for fragment analysis (e.g., size standards, equipment, software, etc.), and highlights the necessity to calibrate methods between studies. At locus *CPG14*, the difference between our estimates of allele sizes and those reported previously were 25–26 bp. This difference was consistent for all four alleles present among the French isolates we genotyped; three of these alleles were also detected in Asturias (Table 3).

In total, we detected 13 multilocus haplotypes among the sample of 189 isolates genotyped (Table 3). The most common haplotype (1) was found in 166 isolates (88%) and was widely distributed among all affected councils. The next most common haplotype (10) included nine isolates (4.8%) and was found only in the southeastern part of Asturias (Fig. 1b). The remaining 11 haplotypes were rare, with each represented by only one or two isolates. Multilocus

Table 3 Multilocus haplotypes defined by alleles at 16 microsatellite loci, vc type and mating type of *Cryphonectria parasitica* in Asturias

Haplo-type	N	Markers used previously in France [a]										Additional markers used in this study [a]							Mating type		
		CPE1	*CPE4*	*CPE5*	*CPE8*	*CPG3*	*CPG4*	*CPE3*	*CPG6*	*I07-650*	*CPG14*	*CpSI 072*	*CpSI 009*	*CpSI 006*	*CpSI 032*	*CpSI 054*	*CPG7*	vc type	*MAT-1*	*MAT-2*	*MAT-1/2*
1	166	*167 (147)*[b]	*250 (229)*	*278 (260)*	*132 (112)*	*235 (214)*	*225 (205)*	*211 (192)*	*263 (244)*	*312 (295)*	*292 (267)*	*348*	*185*	*165*	*464*	*310*	*253*	EU-1	104	55	1
2	2	–[c]	–	–	135	–	–	–	–	–	–	–	–	–	–	–	–	EU–1	1	1	0
3	2	–	–	–	150	–	–	–	–	–	–	–	–	–	–	–	–	EU–1	2		0
4	1	–	–	–	–	–	228	–	–	–	–	–	–	–	–	–	–	EU–1	0	1	0
5	1	–	–	–	–	–	222	–	–	–	–	–	–	–	–	–	–	EU–1	0	1	0
6	1	–	–	–	–	–	–	–	–	315	–	–	–	–	–	–	–	EU–1	0	1	0
7	1	–	–	–	–	–	–	–	–	–	269 (244)	–	–	–	–	–	–	EU–1	0	1	0
8	1	–	–	–	–	–	–	–	–	–	281 (255)	–	–	–	–	–	–	EU–1	0	1	0
9	1	131	–	–	–	253	–	–	–	–	281 (255)	–	–	–	–	–	–	EU–3	0	1	0
10	9	–	–	–	–	–	–	–	–	–	281 (255)	337	167	–	–	–	–	EU–13	9	0	0
11	2	–	–	–	–	–	208	213 (194)	–	–	281 (255)	337	167	–	–	–	–	EU–13	0	2	0
12	1	–	–	–	–	–	–	–	–	–	281 (255)	340	167	–	–	–	–	EU–13	1	0	0
13	1	–	–	–	–	–	–	–	–	–	281 (255)	337	–	–	–	–	–	EU–13	0	1	0

[a] Primers for microsatellite markers were reported previously: *I07-650* (Davis et al. 2005), *CPE1, CPE3, CPE4, CPE5, CPE8, CPG3, CPG4* (Breuillin et al. 2006), *CPG6, CPG14, CPG7, CpSI006, CpSI009, CpSI032, CpSI054* and *CpSI072* (Kubisiak et al. 2007)

[b] Alleles are designated by their fragment size (bp) and include the 19-bp M13-adapter sequence added for fluorescent detection (see Materials and methods for details). Alleles in parentheses are sizes reported by Dutech et al. (2008; 2010) for these same markers and differ from our estimates by 17–21 bp because of the M13 adapter and minor variation in size estimates for the different fragment analysis methods. Alleles without numbers in parentheses were not previously reported in France

[c] Same allele as in haplotype 1

haplotypes were highly correlated to vc types such that each haplotype was associated with only one vc type (Table 3). Multiple haplotypes found within a vc type were typically closely related and, with only one exception (haplotype 11), differed from the most common haplotype within a vc type at only one microsatellite locus (Fig. 2). The presence of closed loops among haplotypes 1, 2 and 3, and 1, 7 and 8 occurs because of multiple mutations at two loci, the order of which cannot be determined. The lack of any other loops in the network is consistent with evolution by mutation only, not recombination.

To compare microsatellite haplotypes found in Asturias to those in France (Dutech et al. 2010), we combined haplotypes that were identical for the ten markers used to genotype populations in France. Four haplotypes (8, 10, 12 and 13) found in Asturias (Table 3) match genotype RE103 for these ten loci, which is one of the most common genotypes in France (Dutech et al. 2010). With these same ten loci, the dominant haplotype (1) differs from RE103 only at locus *CPG14*, and is the same as genotype RE104 which was found in a few isolates in France (C. Robin and C. Dutech, personal communication). Similarly, haplotype 7 differs from RE103 at this same locus, but with a different allele. All other haplotypes differ from RE103 by alleles at two loci (Table 3, Fig. 2).

Discussion

The population of *C. parasitica* in Asturias has a clonal structure dominated by a single vc type (EU-1), mating type (*MAT-1*) and microsatellite haplotype. This almost complete lack of diversity is similar to what has been described for *C. parasitica* in south-eastern Europe, where one clone of vc type EU-12 makes up most of the population (Milgroom et al. 2008). Low diversity for vc types and molecular markers also characterizes the *C. parasitica* population that recently colonized areas in Switzerland north of the Alps (Hoegger et al. 2000). Vc type EU-1 has also been found at relatively high frequencies in locations in Castile and León and Galicia (Montenegro et al. 2008). As areas neighboring Asturias, it is likely that EU-1 isolates in these locations are members of the same clone as in Asturias. However, we cannot determine if vc type EU-1 reported in neighboring areas, or from Catalonia (Trestic et al. 2001) and other parts of Europe

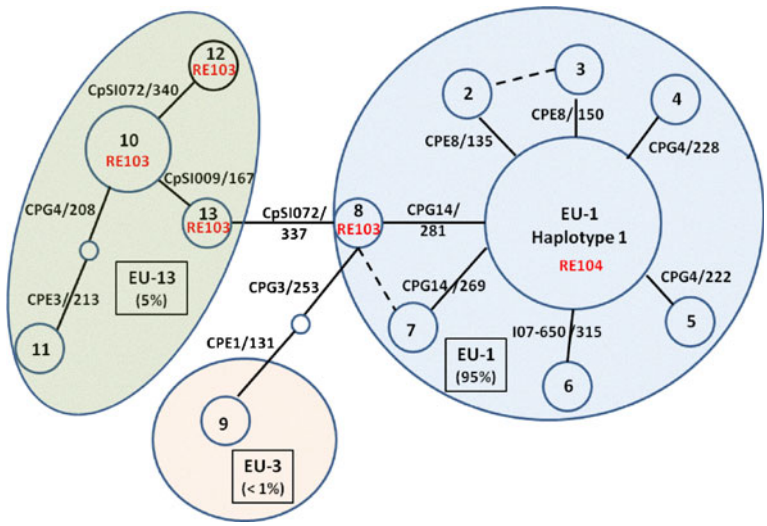

Fig. 2 Haplotype network of *Cryphonectria parasitica* in Asturias based on nine polymorphic microsatellite markers. Each haplotype (defined in Table 3) is represented by a circle and labeled by a haplotype number (1–13). Haplotype 1 was found in 166 isolates, whereas the next most common haplotype (10) was found in nine isolates; all other haplotypes were represented by one or two isolates (Table 3). Lines between haplotypes represent single mutations, which are labeled by locus and allele that are different from haplotype 1. Dashed lines indicate multiple mutations at the same loci, which could have occurred sequentially and not independently. Small circles without haplotype numbers are inferred intermediate haplotypes that were not observed in our sample. Haplotypes are grouped by vc types, with their frequencies in Asturias (Table 1) shown as percentages in parentheses

(Robin and Heiniger 2001) represents the same clone as in Asturias without genotyping isolates from those areas. For example, one EU-1 clone, defined by neutral molecular markers, was found in Sicily and Turkey, but an unrelated clone (defined with the same markers) found in Macedonia was also of vc type EU-1 (Milgroom et al. 2008).

Nearly the entire population in Asturias is identical to or closely related to one (RE103) of the seven most common genotypes in France (Dutech et al. 2010) when haplotypes are defined only by the ten markers used in France. Haplotype 8 in EU-1 and three haplotypes (10, 12 and 13) in EU-13 have the same genotype as RE103 at these ten loci, even though they differed among themselves by vc type and additional microsatellite markers used in Asturias (Table 3, Fig. 2). The dominant haplotype in Asturias (haplotype 1), representing approximately 95% of the population, differs from genotype RE103 at only one of these ten loci and corresponds to French genotype RE104 (C. Robin and C. Dutech, personal communication). Based only on these ten markers, therefore, it would appear that the *C. parasitica* population in Asturias comprises a single clonal lineage. In this scenario, it is possible that *C. parasitica* was introduced from France. Genotypes RE103 and RE104 are members of the C1 clonal lineage, which is common in southeastern France and has strong affinities to the populations in northern Italy that are derived from the original documented introduction (Dutech et al. 2010). Whether *C. parasitica* was introduced from France, Italy or elsewhere cannot be determined in this analysis. Genotyping isolates from other locations would be needed to address this question.

Genotyping with vc type and additional microsatellite markers, however, raises the alternative hypothesis that multiple genotypes were introduced. Vc types EU-1 and EU-13 have different alleles at four *vic* loci (Cortesi and Milgroom 1998). If a single individual of the most common EU-1 genotype, for example, were introduced into Asturias, then mutations at four *vic* loci would have to have occurred to result in vc type EU-13. Although we do not have any estimates of mutation rates at *vic* loci in *C. parasitica*, the widespread occurrence of clones of the same vc type, e.g., across southeastern Europe (Milgroom et al. 2008), suggests that *vic* genotypes are stable, with low mutation rates. Therefore, it would be unlikely that mutations would occur at multiple *vic* loci in Asturias, making the multiple introduction hypothesis more plausible than evolution in situ by mutation alone.

We did not find evidence for recombination from microsatellite genotyping. The haplotype network (Fig. 2) can be explained most simply by a combination of introductions of the different vc types and mutation at microsatellite loci, resulting in rare haplotypes within vc types differing at single loci from the most common haplotypes. The only closed loops in the network (among haplotypes 1, 2 and 3, and 1, 7 and 8) resulted from three alleles occurring at two loci (*CPE8* and *CPG14*, respectively); these form loops because we do not know for sure if these haplotypes arose independently from the most common type or whether mutations occurred sequentially. This does not mean that sexual reproduction is not occurring, only that we cannot detect recombination when there is so little diversity. The presence of both mating types within the dominant clone makes sexual reproduction possible, but would not result in detectable recombination. In Romania, Milgroom et al. (2008) found both mating types and perithecia of *C. parasitica* in the same populations, but no recombinant haplotypes. Similarly, Dutech et al. (2010), also found both mating types present in some populations in France, but no evidence of recombination between lineages.

In our 2005–2007 survey, we detected chestnut blight in 69 of the 78 councils of Asturias. This represents a net increase of nine councils since 2000 (Valdezate et al. 2001). We found chestnut blight in 11 new councils, but we did not find it in two councils where Valdezate et al. (2001) had reported blight earlier (Fig. 1a). This discrepancy could be because disease incidence was low, and chestnut trees are isolated and dispersed. In the council of Muros del Nalón, chestnut blight was detected in 1989–1990 and again in 2005–2007, but not in 2000 (Valdezate et al. 2001). The increased spread of chestnut blight into almost all of Asturias could be a consequence of the abandonment of chestnut forests which allows for more rapid disease progress. The councils in which we did not detect chestnut blight are found in the western part of Asturias, where chestnut forests are less abundant and the movement of chestnut wood is nowadays less intense than in other parts of the region (Valdezate et al. 2001).

One of the long-term goals in this research was to understand the potential for biocontrol with hypovirulence. As in other populations with a low diversity of vc types, conditions in Asturias should be ideal for deploying hypovirulence (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). Although we did not conduct a formal survey for hypovirulence, we found very few white isolates of *C. parasitica*, the hallmark of infection with *Cryphonectria hypovirus 1* (CHV-1). Nonetheless, deployment of CHV-1 might have a good chance of success because 95% of the population has the same vc type and therefore, no barriers to transmission of virus from one individual to another (Cortesi et al. 2001). Other factors also need to be taken into account to predict the success of managing chestnut blight with hypovirulence, such as the amount and effectiveness of the inoculum to establish hypovirulence in an area (Robin et al. 2000). During our surveys, we observed a lack of forest management in the majority of chestnut forests in Asturias, a situation described previously by Valdezate et al. (2001). Managing chestnut blight and the deployment of hypovirulence are more likely to succeed in stands that are being managed intensively, regardless of vc type diversity (Griffin 1986; Milgroom and Cortesi 2004).

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