

## Distribution and Population Structure of the Chestnut Blight Fungus in Romania

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### Abstract

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The occurrence of chestnut blight (*Cryphonectria parasitica*) was studied in 2011–2012 at 13 locations in the main chestnut growing areas of Romania. Infections were detected at four localities. The symptoms and the fungus were detected on European chestnut (four localities) and also on oak trees (two localities). A total of 89 isolates of *C. parasitica* were isolated and characterised. Based on canker and isolate morphology (culture morphology and the Bavendamm test), both virulent and hypovirulent samples were isolated; hypovirulent isolates were found at only one locality. Two vegetative compatibility types corresponding to EU-12 and EU-2 were identified among isolates. Both mating types were found, with a dominance of *MAT-1* in southern Romania and *MAT-2* in northern Romania.

**Keywords:** *Cryphonectria parasitica*; European chestnut; oak; hypovirus; vc types; mating types

The European chestnut (*Castanea sativa* Mill.) is a widespread broad-leaved tree species that has an invaluable historical, cultural, economic, and environmental role in Europe. In Romania it can be found mainly in the Oltenia region in the southwest of Romania and in Maramureş in the northwest of the country. In Oltenia, old chestnut forests exist, mainly near old monasteries, groves or small orchards (BOTU 2010). Isolated chestnut trees are common in the same area of Oltenia and can also be found in Transylvania. In the Maramureş province, chestnut forests are located near the town of Baia Mare and in this region several very old trees are present (first reported in 1642) (BOTU 2010).

*Cryphonectria parasitica* is the causal agent of chestnut blight, a severe disease responsible for the

devastation of chestnut stands in North America and Europe. In Romania the disease was first noticed in 1984 (FLOREA & POPA 1989; BOLEA *et al.* 1995). The fungal populations in stands around Baia Mare in northwest Romania have been studied previously (RADÓCZ 2001; MILGROOM *et al.* 2008). RADÓCZ (2001) detected only one vegetative compatibility (vc) type (EU-12) among 17 isolates in three local stands in Baia Mare, which all had orange culture morphology. MILGROOM *et al.* (2008) investigated 27 isolates from three local stands around Baia Mare. All isolates were compatible with vc type EU-12, except for one isolate, which was classified as EU-2. Both mating types were detected with predominance of *MAT-1*. No data concerning the occurrence and population structure of *C. parasitica* in other parts of the country have been reported.

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The predominance of vc type EU-12 and mating type idiomorph *MAT-1* is generally observed in southeast Europe – Bulgaria (MILGROOM *et al.* 2008), Macedonia, and Greece (SOTIROVSKI *et al.* 2006). In the countries surrounding Romania low vc type diversity was observed. In Bulgaria two vc types (EU-12, EU-10) were determined (MILGROOM *et al.* 2008), and in Ukraine only one vc type (EU-12) was detected (RADÓCZ 2001). A higher diversity was found in Hungary, which is the neighbouring country to the west, with 18 vc types (RADÓCZ 2001).

Information on the population structure of *C. parasitica* is important for the success of biological control of chestnut blight with transmissible hypovirulence (HOEGGER *et al.* 2000). In Europe, hypovirulence in *C. parasitica* is caused by a RNA mycovirus, named *Cryphonectria hypovirus 1* (HILLMAN *et al.* 2000). The hypovirus may be transmitted vertically into asexual spores and horizontally among fungal individuals. Horizontal transmission of hypovirulence only occurs after hyphal anastomosis and the mixing of cytoplasm of one individual with another. Vegetative incompatibility reduces hyphal anastomosis among individuals

and thus restricts hypovirus transmission. Vc type diversity is one of the factors affecting hypovirus spread in *C. parasitica* population and success of biological control (MILGROOM & CORTESI 2004). *C. parasitica* populations can be very diverse in their genetic structure. In China and Japan, the diversity is very high (WANG *et al.* 1991), and in North America, it ranges from clonal to highly diverse (MILGROOM *et al.* 1992; MILGROOM & LIPARI 1995). In Europe, the diversity of chestnut blight fungus is generally lower than in North America (CORTESI *et al.* 1996, 1998).

Biological control is the fundamental measure for the ecological restoration of infected chestnut stands. This was tested in Maramureş (2005–2008), most of the treated cankers were healed, and from 2007 the natural spread of virus to untreated trees in experimental plots has also been observed. Managed stands will be biologically treated (minimum 50 inocula/ha) for several years (BOLEA *et al.* 2010).

The aims of this study were: (i) to determine the occurrence of *C. parasitica* in Romania; (ii) to investigate the presence of hypovirus in *C. parasitica* isolates; (iii) to characterise *C. parasitica* populations

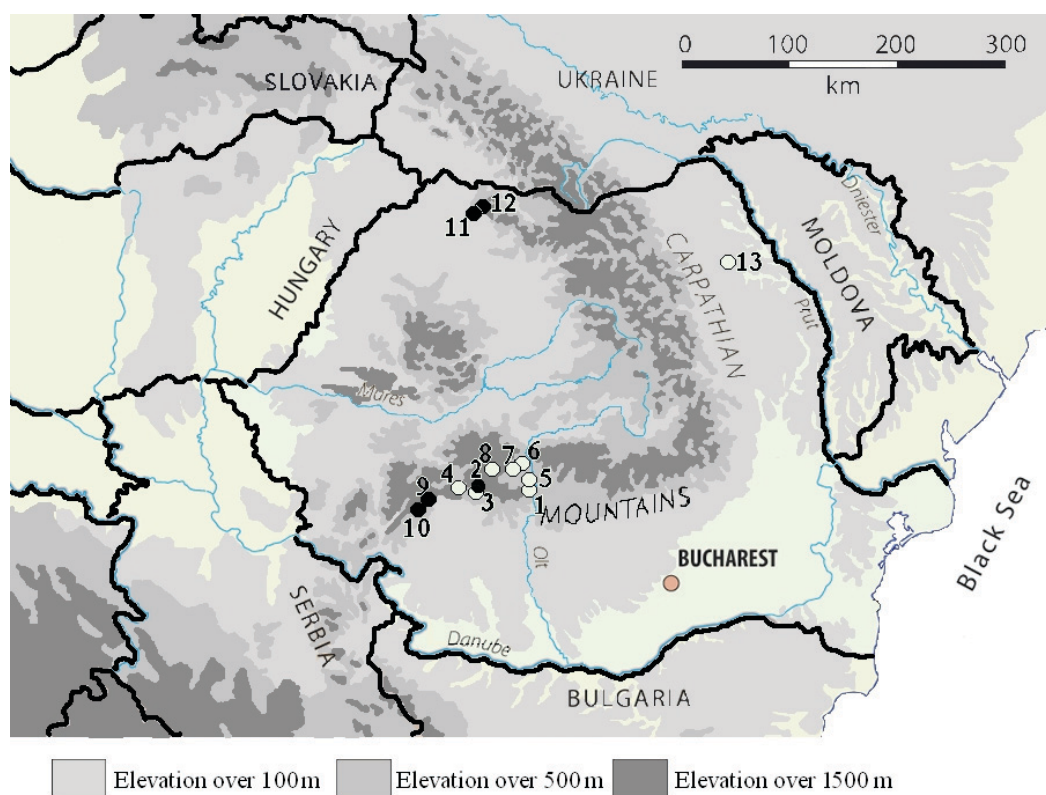


Figure 1. Localities in Romania where chestnut blight was surveyed and *Cryphonectria parasitica* samples were collected. Numbers 1–13 in the map correspond with the names of localities given in Tables 1–3. Black dots are localities where the presence of chestnut blight disease was recorded, white dots indicate disease-free localities

in respect to the diversity of vegetative compatibility (vc) types and mating types. This study should significantly contribute to understanding the chestnut blight population in the part of Europe, where data concerning fungal populations are lacking.

## MATERIAL AND METHODS

**Study area.** In the years 2011–2012, European chestnut stands in Romania were evaluated for the occurrence of the fungus *Cryphonectria parasitica*. Two geographically separate areas were studied; the first area was located in the Oltenia province in south-western Romania, where ten locations were visited, and the second area was situated in the northern part of the country in the Maramureş province, where two chestnut stands were evaluated. In total, 13 locations of European chestnut were evaluated, including Iasi in the Moldova region (Table 1). At all

locations, sampling of *C. parasitica* was performed from chestnut blight cankers using methods reported previously (CORTESI *et al.* 1996). Only one randomly selected canker per tree was sampled. The number of collected samples depended on the size of stand and/or the number of infected trees (range 4–43 per stand). The numbers of examined trees and collected samples are shown in Tables 1 and 2.

The following symptoms were evaluated on trees: dry leaves that remained attached on the trees even after they got dry; distinct colour changes and depressions on the smooth stems and the branches; cracks and peeling of bark; presence of stem and root shoots; the fan-shaped yellow mycelium visible under bark; on the bark surface red-orange stromata of *C. parasitica*.

**Fungal isolates.** Bark samples (4–5 cm long) were removed from the margin of chestnut blight cankers using a knife, surface-sterilized in 0.15% NaClO for 20 min and subsequently washed in distilled water. Small pieces of bark tissue (ca 0.5 × 0.5 cm) were

Table 1. Description of investigated localities

No	Name of locality	Coordinates	Type of stand	Age of trees	No. of examined chestnut trees/oaks	Presence of chestnut blight cankers*
1	Rm. Vâlcea (SCDP Valcea)	45°8'27.56"N 24°22'17.26"E	orchard	4–15	330	no
2	Horezu I – North of the Horezu Monastery	45°10'28.91"N 24°00'31.76"E	forest	80–120	30	yes – A
3	Horezu II – near Horezu Monastery	45°10'10.99"N 24°00'21.18"E	orchard	30–350	25	no
4	Polovragi	45°11'32.09"N 23°47'28.69"E	forest	60–350	30	no
5	Dăeşti	45°10'19.82"N 24°22'54.26"E	orchard	80–120	11	no
6	Mănăstirea Turnu – near Călimăneşti	45°17'29.23"N 24°18'8.95"E	orchard + forest	80–150	15	no
7	Frăsinei	45°14'22.75"N 4°15'44.48"E	orchard	40–120	6	no
8	Bistriţa	45°11'18.07"N 4°02'14.82"E	orchard	200–350	15	no
9	Gureni	45°06'41"N 23°02'02"E	orchard + forest	25–150	20/10	yes – A
10	Topesti – Nereaz – Gornoviţa	45°04'21"N 22°57'15"E	orchard + forest	30–350	30	yes – A
11	Baia Mare I Valea Roşie-Morgău	47°40'19.74"N 23°33'27.20"E	forest	30–150	60/15	yes – A, H
12	Baia Mare II Valea Usturoiului	47°41'0.68"N 23°34'20.85"E	forest	30–150	30	yes – A
13	Iasi (SCDP Iaşi) – Sârca	47°13'50.26"N 27°10'47.52"E	isolated trees	12–15	2	no

Numbers 1–13 correspond to locations in Figure 1; \*type of cankers at infected localities: A – active, H – hypovirulent

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placed on 3% malt agar and incubated at 25–27°C in the dark. Each bark sample with fruiting bodies was examined under a binocular microscope for the presence of perithecia.

**Characterization of *Cryphonectria parasitica* cankers and isolates.** The determination of virulence and the infection of *C. parasitica* with hypovirus were based on visual evaluation of cankers in the field, the morphological features of the colony grown on potato dextrose agar (PDA), the Bavendamm test and hypovirus specific PCR.

The visual evaluation of normal and hypovirulent cankers was performed according to BIRAGHI (1953), GRENTÉ (1965), ELLISTON (1982), and MILGROOM and CORTESI (2004). Normal, hypovirus-free strains of *C. parasitica* invade wounds in the bark of chestnut trees, destroy the cambial tissues, and cause cankers that are sunken in appearance. Cankers expand and girdle the stem, killing all parts of the tree distal to the canker. Hypovirus infected individuals cause swollen, superficial cankers (MILGROOM & CORTESI 2004). Sprouting from bark below the infection is either absent or greatly reduced. The bark surface is cracked lengthwise and roughened, in older infections it becomes blackened and broken into smaller scales (ELLISTON 1982). According to GRENTÉ (1981) and GRENTÉ and BERTHELAY-SAURET (1969a,b), virulent and hypovirulent isolates of *C. parasitica* are morphologically different in culture and distinguishable to the naked eye: the mycelium of the virulent isolates is white, later turning to yellow or orange-yellow and about 96–140 h after subculturing, abundant, globose red-orange pycnidia are observed. In the hypovirulent isolates, the mycelium remains white and the production of pycnidia is low.

All *C. parasitica* isolates for the assay were incubated at 20–22°C under diffuse daylight on the laboratory bench for 21 days. Isolates with white culture mor-

phology were subjected to the phenol oxidase test and tested for the presence of the hypovirus. The phenol oxidase test was performed on agar medium containing tannic acid as described by RIGLING *et al.* (1989). A dark discolouration of the agar medium was considered as phenol oxidase-positive, which indicated a virulent reaction, whereas no colour change indicated a hypovirulent reaction (RIGLING *et al.* 1989).

Total RNA was extracted using a NucleoSpin RNA Plant (Macherey-Nagel, Düren, Germany) according manufacturer's protocols. First strand cDNA was synthesised from total RNA using random hexanucleotide primers according to the Super cDNA synthesis kit protocol. PCR was performed using specific primers EP713-5 and R2280 ORFs to amplify part of the ORF A (ALLEMANN *et al.* 1999). The detection of PCR products was visualised on 1% agarose gels.

Both positive and negative controls were used for the determination of hypovirus presence; the same controls were used for visual evaluation of the culture morphology, Bavendamm test and hypovirus detection by conventional PCR. As a negative control the virulent strain EU 12 (SA 16 from European vc tester database; Tonara, Italy) (CORTESI *et al.* 1998) was used and as a positive control hypovirulent strain R-5 (Rezi, Hungary) (ALLEMANN *et al.* 1999; RADÓCZ 2001).

**Vegetative compatibility test.** Isolates of *C. parasitica* were assayed for vc type as described by CORTESI *et al.* (1996). Isolates less than 10 days old were used for vc tests, which were performed on a potato dextrose agar green (PDAG) medium described by POWELL (1995). The vc type was assessed after 5–7 days according to the merging/barrage response, using 31 European tester strains of *C. parasitica* (CORTESI *et al.* 1998).

**Mating type assays.** In total, 78 isolates were assayed for mating type using a PCR assay with primers M1-GS1n and M1-GS3rev for *MAT-1* and primers M2GS3 and

Table 2. List of locations where chestnut blight was recorded, number of collected samples, and presence of fruiting bodies of *Cryphonectria parasitica*

No.	Locality	Year of sampling	No. of samples collected from chestnut/oak	No of successful isolations	Presence of fruiting bodies
2	Horezu I	2011	12/0	12	pyc (7)
9	Gureni	2011	13/2	15	pyc (3)
10	Topesti	2011	15/0	15	pyc (8)
11	Baia Mare I Valea Rosie	2012	42/1	43	pyc, pt (6)
12	Baia Mare II Valea Usturoiului	2012	4/0	4	pyc (2)

Numbers in first column correspond to locations in Figure 1; pyc – pycnidia; pt – perithecia; numbers in parenthesis represent the number of positive bark samples for individual fruiting bodies



gs1-d-1 for *MAT-2*. Isolate culture, DNA preparation, and PCR assays were performed as described previously (MARRA 1998 in MCGUIRE *et al.* 2004). Strains of *C. parasitica* from the Swiss Federal Research Institute; WSL, M1297 and M1115, were used as the control strains for *MAT-1* and *MAT-2*, respectively (RIGLING 1995).

## RESULTS

### *Field survey, material sampling and isolation.*

Thirteen localities in Romania were investigated in total (Figure 1 and Table 1). Chestnut blight was found in the following four localities: Horezu, Gureni, Topesti, Baia Mare (two different stands; Valea Roşie-Morgău and Valea Usturoiului) (Table 1). Three localities (Horezu, Gureni, Topesti) are situated in southern Romania and one (Baia Mare) in the north (Figure 1). The symptoms of the disease were observed on *Castanea sativa* and *Quercus petraea* host trees. Apart from chestnuts, infected oaks were recorded only in two localities (Gureni, Valea Roşie-Morgău).

Cankers were observed in different parts of the trees (on branches, stem, and stem basis). The symptoms typical for the chestnut blight disease were noticed. The symptoms on infected oaks were similar to those on chestnut. Because of the strongly wrinkled bark, the cankers were difficult to recognise on oaks. But the presence of fruiting bodies of the fungus and mycelium under the peeled bark allowed the identification of the disease.

Based on canker morphology, both virulent and hypovirulent cankers were observed. The observed hypovirulent cankers were not only treated healing

cankers, but also naturally hypovirulent. Virulent cankers were present at all localities with the presence of chestnut blight but hypovirulent cankers were only recorded at one locality at Baia Mare stand Valea Roşie-Morgău.

During the studied period, 89 samples from affected trees were collected (86 from chestnuts; 3 from oaks). All collected samples were identified as *C. parasitica*, based on symptoms and microscopic investigation of fruiting bodies and all yielded the isolate of this fungus.

The bark samples from infected trees with stromata were examined for the presence of pycnidia and perithecia; pycnidia were observed in all samples, but perithecia were only present at Baia Mare (only in the stand Valea Roşie-Morgău) (Table 2).

**Characteristics of *C. parasitica* isolates.** At the beginning of culturing, all isolates had white mycelium. Subsequently, the majority of the isolates turned light yellow or orange-yellow and some weeks later, to red-orange and sporulated abundantly. All isolates from the Horezu, Gureni, Topesti, Baia Mare Valea Usturoiului sites had the orange culture morphology described by GRENTÉ (1981) and based on this phenotype, they were considered to be hypovirus-free. In twelve isolates from the locality Baia Mare Valea Roşie-Morgău mycelium remained white or creamy in colour with little or no sporulation. According to the visual evaluation of culture morphology these twelve isolates were considered to be putatively hypovirus-infected.

In phenol oxidase activity two isolates produced no colour reaction when grown on tannic acid medium, the other white and creamy isolates showed a moderate or strong phenol oxidase reaction.

Table 3. Distribution of vegetative compatibility (vc) types and mating types of *Cryphonectria parasitica* samples

No	Name of locality	Vc type			Mating type		
		N	EU 12	EU 2	N	<i>MAT-1</i>	<i>MAT-2</i>
2	Horezu I	12	12	0	12	12	0
9	Gureni	15	15	0	14*	11	4
10	Topesti	15	15	0	14	13	1
11	Baia Mare I Valea Rosie	43	42	1	34*	13	28
12	Baia Mare II Valea Usturoi	4	4	0	4	0	4
Total		89	88	1	78		

Numbers in the first column correspond to locations in Figure 1; \*1 isolate in location Gureni (G7) and 7 isolates in location Baia Mare (BM4, BM7, BM10, BM15, BM19, BM34, BM42) were MAT 1/2 putative heterokaryons for mating type (MCGUIRE *et al.* 2004), were counted for both *MAT-1* and *MAT-2*

Total RNA was extracted from 78 *C. parasitica* isolates from all studied sites and used to generate cDNA. The presence of *Cryphonectria hypovirus* 1 was tested by PCR using specific primer pairs for the hypovirus. No isolate amplified a PCR product of expected size.

**Vegetative compatibility (vc) test and vc type diversity.** Each isolate was assigned unambiguously to a vc type. Among the examined set of 89 isolates, two vc types (EU-12 and EU-2) were detected (Table 3). All isolates except for one belonged to the vc type EU-12. Only one isolate collected from the Baia Mare stand Valea Roşie-Morgău was compatible with the EU-2. No vc type diversity was observed from southern Romania and in the north, more than one vc type was only detected in one location.

**Mating type.** In total, 78 *C. parasitica* isolates (87% of all isolates) were assayed for mating type. Both mating type idiomorphs (*MAT-1* and *MAT-2*) were identified. Both of them were detected within a single isolate from Gureni (isolate G7) and in seven isolates from Baia Mare (BM4, BM7, BM10, BM15, BM19, BM34, BM42). The predominance of *MAT-1* (24 from 40 isolates) was detected in three locations in southern Romania, whereas *MAT-2* predominated in the north, in the stands around Baia Mare.

## DISCUSSION

The diversity of *C. parasitica* in Romania is among the lowest reported for this species. Almost all isolates were of vc type EU-12 (98.9%). Mating type determination indicates the presence of both idiomorphs, *MAT-1* and *MAT-2*; mating type ratios differed significantly from a 1:1 distribution in all studied locations. Hypovirulent cankers were reported only at one locality (Baia Mare), but the presence of *Cryphonectria hypovirus* was not confirmed by PCR assay.

Clonality appears to be a common feature of *C. parasitica* outside the major disease areas (HOEGGER *et al.* 2000). In Bosnia-Herzegovina, only one vc type was detected in the eastern region, which was compatible with EU-12. This vc type is also dominant in the southwestern parts of the country (TRESTIC *et al.* 2001). In Ukraine, all isolates (from five locations) were assigned to vc type EU-12 (RADÓCZ 2001). Only one vc type (EU-12), which is dominant all over the country, was found in northern Greece (PERLEROU & DIAMANDIS 2006). The vc type EU-12 is also the

only or the dominant type among *C. parasitica* populations in southeastern and eastern Slovakia, which is the geographical northern border of chestnut blight occurrence (ADAMČÍKOVÁ *et al.* 2006, 2012; JUHÁSOVÁ *et al.* 2006).

Likewise, in North America, geographically disjunctive founder populations of *C. parasitica* outside the natural range of chestnuts have also a lower genotypic diversity than populations in the centre of the host populations (LIU *et al.* 1996; MCGUIRE *et al.* 2005).

Both mating type idiomorphs, with a predominance of *MAT-1*, were detected in northern Romania in a previous study (MILGROOM *et al.* 2008). In the joint stand in previous and present study (Baia Mare Valea Rosie) *MAT-2* was also detected. However, idiomorph *MAT-1* was dominant in southern Romania. These locations are closer to the Bulgarian border, on the southeast slope of the Carpathian Mountains and there are no geographical barriers that limit migration. The dominant vc type in north Bulgaria is EU-12 and only idiomorph *MAT-1* was recorded (MILGROOM *et al.* 2008). The populations in southern Romania are similar to those in the rest of south-eastern Europe. We speculate that the disease was introduced there from northern Bulgaria. Other possible sources of disease in southern Romania might be seedlings transported from the north of the country (personal communication with local forestry people), where the disease first occurred and the same vc type (EU-12) and mating type *MAT-1* (MILGROOM *et al.* 2008) were dominant. The origin of the mating type *MAT-2*, which was found at a low frequency in two of the southern populations, is not known but it could have emerged by selfing, which would result in the production of progeny of both mating types (HOEGGER *et al.* 2000).

Eight isolates were *MAT-1/2* and might represent heterokaryons for mating type (MCGUIRE *et al.* 2004, 2005). Similarly, MILGROOM *et al.* (2008) detected both mating type idiomorphs in the same stand in Baia Mare, Valea Rosie-Morgău. This can be explained by recombination at the MAT locus; mating type heterokaryons have been commonly found in *C. parasitica* in the USA (MILGROOM *et al.* 2008).

In a population with frequent sexual reproduction, it is expected to find a mating type ratio approaching 1:1 (HOEGGER *et al.* 2000). Only in the Baia Mare, Valea Rosie-Morgău location the mating type ratio was the closest to this and this was the only stand where sexual fruiting bodies were detected. The dominance of one mating type in all other populations clearly

indicates the importance of asexual reproduction in the studied populations; sexual reproduction played only a minor role, if any, in these populations.

The results suggest and confirm that *C. parasitica* is disseminated almost exclusively by asexual conidia or mycelial fragments after its introduction into chestnut trees. Nevertheless, the potential for sexual reproduction exists in two other populations (Gureni, Topesti), where both mating types are present.

Clonal populations with little or no diversity within vegetative incompatibility should be ideal candidates for biological control (LIU *et al.* 2000). Based on laboratory studies (LIU & MILGROOM 1996; CORTESI *et al.* 2001), vegetative incompatibility is thought to be a major constraint on the spread of hypoviruses in the field (ANAGNOSTAKIS *et al.* 1986; MILGROOM 1999). The incidence of the hypovirus in southeastern Europe is variable and in most locations it is low (GURER *et al.* 2001; SOTIROVSKI *et al.* 2006). Variation might represent the lack of introduction of viruses to some locations, where they might potentially spread rapidly in clonal populations once they are introduced (MILGROOM *et al.* 2008). The time between the introduction of *C. parasitica* and the natural appearance of the hypovirus in Europe is about 20 to 30 years (ROBIN & HEINIGER 2001).

The *C. parasitica* populations in southern Romania were all virulent, based on the cankers and isolate morphology and hypovirus specific PCR. Only at one locality in northern Romania (Baia Mare) both virulent and hypovirulent cankers (based on canker morphology) were recorded. Few isolates showed white culture morphology and the phenol oxidase test indicated hypovirulent reactions for two isolates. Culture morphology and phenol oxidase activity can give a broad idea about hypovirulence but it did not always indicate the presence of hypovirulence (AKILLI *et al.* 2013). However it does not provide any other information about the hypoviruses (AKILLI *et al.* 2013). In our study the PCR assays were negative in all cases. We expected the hypovirus presence at least in two isolates (according phenol oxidase tests). The results may suggest that the hypovirus is present in these isolates but its concentrations are below the detection limits when using the PCR assay. At location Baia Mare, where the white isolates were determined, the experimental inoculation of hundreds of virulent cankers was performed during four years (2005–2008) (BOLEA *et al.* 2010). For the experiments, two Greek strains from Forest Research Institute Thessaloniki were used for conversion of

Romanian strains to hypovirulent, which have been used for field treatments (CHIRA *et al.* 2005). Most of the treated cankers were healed; the efficiency of canker healing was 70–90%, depending on canker size (BOLEA *et al.* 2010). The natural spread of the hypovirus to untreated trees in experimental plots was also observed starting from 2007 (BOLEA *et al.* 2010). The hypovirus established only in one stand and appeared to spread slowly in the *C. parasitica* population in this area because it was detected only in a stand where the treatment was performed and was detected neither in other evaluated locations nor in the geographically closest stands. Therefore, it might be appropriate to treat more stands with hypovirulent isolates, to promote hypovirulence in all stands where chestnut blight occurs, which might have a positive impact on the recovery of chestnut stands. Sexual reproduction and a potential increase in vc type diversity could create an obstacle for biological control and spread of the hypovirus. Therefore, frequent monitoring of vc type diversity and hypovirus spread is advisable.

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