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Temporal and spatial genetic population structure of *Cryphonectria parasitica* and its associated hypovirus across an invasive range of chestnut blight in Europe

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Running title: European chestnut blight fungus and CHV1

Abstract

Chestnut blight has spread throughout Europe since the introduction of its causal agent *Cryphonectria parasitica* over 70 years ago. In our study, we have analysed diversity of vegetative compatibility (vc) and microsatellite genotypes of *C. parasitica*, as well as sequence diversity of *Cryphonectria hypovirus 1* (CHV1) in six populations from Switzerland, Croatia and North Macedonia. Resampling of local populations that were already investigated more than a decade ago allowed us to analyse the spatial and temporal population structure across an invasive range of the pathogen in Europe. Regardless which genetic marker was used, the over 60 year-old Swiss and Croatian populations had a high population diversity, while younger North Macedonian populations were mostly clonal. These diversity differences between the investigated populations remained stable over time. A high diversity of CHV1 was observed in all three countries, with North Macedonian strains forming a separate cluster from strains obtained in other countries. No correlation between vc diversity and CHV1 prevalence was observed, suggesting a well-established and maintained natural hypovirulence in all countries, further corroborated by an observed increase in genetic diversity of Croatian *C. parasitica* populations over time, without collapse of CHV1 prevalence.

Keywords: biological control, phytopathogenic fungus, population genetics, RNA virus

Introduction

The number of invasive forest pathogens has increased exponentially in the last decades resulting in significant disturbance to ecosystems and severe socio-economic impact (Aukema et al. 2011). Nowadays, invasive pathogens are the main cause of emerging

infectious diseases on forest trees (Santini et al. 2013). Dutch elm disease, chestnut blight, and more recently, ash dieback are among the large number of striking examples of phytopathogenic invasive fungi in forest ecosystems (Anderson et al. 2004; Liebhold et al. 2012; Gross, Hosoya, and Queloz 2014).

The causal agent of chestnut blight is the ascomycete fungus *Cryphonectria parasitica* (Murrill) Barr, probably one of the best known invasive fungal pathogens (Anagnostakis, 1987). Native to Asia, *C. parasitica* was introduced into North America and Europe in the 20th century where it caused severe disease epidemics on the native chestnut species (Anagnostakis, 1987). In the eastern USA, chestnut blight killed nearly all native American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) throughout its 3.6-million ha distribution range. In Europe, the first symptomatic chestnut trees (*C. sativa* Mill.) were observed in 1938 in Italy near the main commercial port of Genoa (Robin and Heiniger, 2001). From there, a central European population of the fungus was established in northern Italy (Milgroom *et al.*, 2008), which then rapidly expanded into neighbouring countries, including eastern France, Switzerland, Slovenia and Croatia (Heiniger and Rigling 1994). This central European population was most likely the source for further spread of the disease to other chestnut-growing regions in Europe, e.g. the Balkans (Stauber, Prospero, and Croll 2020). Additional introductions of *C. parasitica* into the natural distribution area of European chestnut impacted south-western France (Dutech et al. 2012) and the Caucasus region (Prospero et al. 2013). In Europe, damage caused by chestnut blight gradually became less severe due to the establishment and spread of *Cryphonectria hypovirus 1* (CHV1), an effective biological control agent of *C. parasitica.* CHV1 was most likely introduced to Europe together with its fungal host and has subsequently spread following *C. parasitica* (Heiniger and Rigling 1994; Peever et al. 1998; Liu and Milgroom 2007; Feau et al. 2014). CHV1 reduces virulence, sexual and asexual reproductive ability, and pigmentation of infected *C. parasitica* strains, a

phenomenon called hypovirulence, which enables the recovery of the diseased chestnut trees (Peever et al. 2000; Hillman and Suzuki 2004; Bryner and Rigling, 2012). CHV1, as a typical RNA virus, accumulates mutations rapidly, producing many viral variants over a short period of time (Gobbin et al. 2003; Mlinarec et al. 2018a). Several genetically distinct subtypes of CHV1 occur in Europe, named according to the region of first detection (Allemann et al. 1999; Sotirovski et al. 2006; Robin et al. 2010; Mlinarec et al. 2018a; Rigling et al. 2018). The Italian subtype is the only one occurring across a large area in southern and south-eastern Europe (Feau et al. 2014; Krstin et al. 2019; Robin et al. 2010; Sotirovski et al. 2006).

CHV1 does not have an extracellular phase and is transmitted both vertically via asexual spores (conidia) and horizontally via hyphal anastomoses between infected and uninfected mycelia (Hillman and Suzuki 2004). However, horizontal transmission is restricted by the vegetative incompatibility system of *C. parasitica* (Cortesi et al. 2001). The incompatible reaction triggered by the vegetative incompatibility system constrains cytoplasmic exchange, thus restricting virus transmission. Vegetative incompatibility in *C. parasitica* is determined by at least six unlinked di-allelic *vic* loci, which define 64 *vic* genotypes or vegetative compatibility (vc) types (Cortesi and Milgroom 1998). A high vc type diversity is expected to limit the horizontal spread of the hypovirus within *C. parasitica* populations and therefore represent an obstacle for biological control of chestnut blight using hypovirulence (Rigling and Prospero 2018).

The genetic diversity of European *C. parasitica* populations has been characterized by typing isolates either at the *vic* loci (e.g. Cortesi et al. 1998; Krstin et al. 2008; Robin et al., 2000; Sotirovski et al. 2004) or at microsatellite loci (e.g. Dutech et al. 2010; Ježić et al. 2012; Milgroom et al. 2008; Prospero and Rigling 2012). Both markers types show that *C. parasitica* populations at the expanding front of the disease are highly clonal, whereas older and well-established populations, such as those that were closest to the areas of first

introduction of *C. parasitica* into Europe, are more diverse. In North Macedonia, Greece and Bulgaria, for example, a single vc type (EU-12) was found to be dominant (Risteski et al. 2013; Sotirovski et al. 2004). On the other side, in over 60 years old established *C. parasitica* populations in Croatia and Switzerland up to 24 different vc types were found (Bryner and Rigling 2012; Ježić et al. 2018).

Recent investigations have shown that the genetic structure of European *C. parasitica* populations can change drastically in a relatively short time period, due to the appearance of novel vc types (Ježić et al. 2018). For example, over a period of 20 years an increase in the number of vc types was observed in Germany (Peters et al. 2014). The authors argued that several introductions of *C. parasitica* occurred, resulting in an increased diversity of vc types in local populations. In two *C. parasitica* populations from Croatia, the number of vc types more than doubled within ten years, from eight to 20 (Krstin et al. 2008). The observed changes were explained by immigration of new vc types from other populations and generation of new vc types by sexual recombination (Ježić et al. 2018). Noteworthy, CHV1 prevalence in Croatia, regardless of the increase in vc type diversity, increased in a population which previously had low CHV1 prevalence, and decreased in a population which previously had high CHV1 prevalence (Ježić et al. 2018). At the disease front, the prevalence of hypovirulence may be variable, but is generally low, as seen in south-eastern Europe (Sotirovski et al. 2006; Radócz 2001) and northern Switzerland (Hoegger et al. 2000).

In this study, we investigated genetic diversity of *C. parasitica* and associated CHV1 in six populations across the pathogen's invasive range from central to south-eastern Europe i.e. Switzerland, Croatia and North Macedonia. In all three countries, the resident *C. parasitica* populations have been characterized in previous years, which allows investigation of temporal patterns of population change. Moreover, these populations had several distinct characteristics which made them particularly well suited for comparison across the *C.*

125 *parasitica* range in Europe (Table 1). For example, the first appearance of the disease and
126 natural hypovirulence occurred in the three studied countries over a course of almost 50 years.
127 In Switzerland *C. parasitica* was first recorded in 1948 and hypovirulent isolates were
128 identified in 1975 (Heiniger and Rigling 1994). In Croatia these events occurred in 1955 and
129 1978, and in North Macedonia in 1975 and 1995, respectively (Heiniger and Rigling 1994;
130 Robin and Heiniger 2001). Although this ~20 years of delay might be biased by the
131 methodology of hypovirus detection, it is reasonable to assume that virulent isolates spread
132 faster into new areas due to their more vigorous reproductive capacity. Since the Swiss
133 populations are the oldest, and those in North Macedonia the youngest, we expected to detect
134 a persistent negative gradient in *C. parasitica* and CHV1 diversity, as well as in CHV1
135 prevalence, from Switzerland to North Macedonia, a pattern which has been hinted at by
136 previous studies (Bryner et al. 2012; Milgroom et al. 2008). These studies, however, used
137 different markers and targeted either the fungus or the hypovirus. Here, we combined
138 microsatellite and *vic* genotyping for the fungus and single-nucleotide polymorphisms (SNPs)
139 for the virus. Using both classical markers important for the dissemination of CHV1 within *C.*
140 *parasitica* populations (vegetative compatibility (vc) types), as well as microsatellite markers
141 allowed the characterization of the six *C. parasitica* populations, two from each of the
142 investigated countries. Beyond that, the inclusion of CHV1 analysis (including changes in
143 prevalence in populations and SNP analysis) provided deeper insight into the spatial and
144 temporal development of the *C. parasitica*-hypovirus pathosystem in Europe.
145
146 **Materials and Methods**
147 ***Cryphonectria parasitica* sampling and isolation**
148 Sixty to eighty randomly chosen chestnut blight cankers per location were sampled in
149 May 2014 in six *Castanea sativa* stands in Croatia, North Macedonia and Switzerland (Table

1). All sample sites were coppice forests with ~15-year-old chestnut sprouts. We collected three bark samples per canker (upper margin, middle, and lower margin of the canker). Bark samples were extracted using a bone marrow biopsy needle (diam. 2 mm); the needle was sterilized between each sampling by dipping in 96% ethanol and flaming. In the laboratory, bark samples were surface sterilised using 70% ethanol and placed on potato dextrose agar (PDA; 39 g/L, BD Difco™ Sparks, MD, USA). Plates were incubated in the dark at room temperature until mycelial growth was observed. Small pieces of the outgrowing colonies were then transferred to a new Petri dish containing PDA and incubated for ten days in a climate chamber at 24 - 25 °C, in the dark and then transferred to laboratory bench at room temperature for an additional five days. After this period, cultures were classified as hypovirus-free if they displayed orange culture morphology and hypovirus-infected if they displayed white culture morphology (Bissegger et al. 1997; Robin et al. 2010). Only one *C. parasitica* isolate per canker was selected for further analysis. If white isolates were recovered from a canker, the canker was considered hypovirus-infected and one randomly selected white isolate was used for further analysis.

DNA and RNA extraction

For nucleic acid extraction, a small plug of PDA with *C. parasitica* mycelium was transferred to a Petri dish with cellophane overlaid onto PDA (Hoegger et al. 2000), and grown for five days in the dark at 24˚C. The mycelium was then scraped from the cellophane and fungal DNA was extracted using two commercially available DNA extraction kits: OmniPrep for Fungi (G Biosciences, Saint Lewis, MO, USA) or King Fisher™ Flex Purification System (Thermo Fisher Scientific, Vilnius, Lithuania). Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). All extractions were performed according to manufacturers' instructions.

175 **Vegetative compatibility type and microsatellite genotype determination**

176 The vc type of each isolate was determined by paring it with EU vc type tester strains
177 (Cortesi and Milgroom 1998) or by PCR-based *vic* genotyping as described by (Mlinarec et
178 al., 2018b; Short et al., 2015). The *vic* genotype of each isolate was assigned to a EU vc type
179 as defined by Cortesi and Milgroom (1998). The vc type obtained by PCR was then
180 confirmed by traditional co-culturing of each isolate with the corresponding EU vc type tester
181 strain (Cortesi and Milgroom 1998).

182 All *C. parasitica* isolates were genotyped at 10 microsatellite loci using the primers
183 pairs CPG3, CPG4, CPG6, CPG14, CPE1, CPE3, CPE4, CPE5, CPE8 (Breuillin, Dutech and
184 Robin 2006), and I07-650 (Milgroom *et al.*, 2008). PCR reactions were performed as
185 described by Prospero and Rigling (2012) and the resulting electropherograms were analysed
186 with the software GeneMapper® 5 (Applied Biosystems™ Waltham, MA, USA). The
187 detected haplotypes were named according to Prospero and Rigling (2012). Only isolates with
188 clearly and unambiguously determined vc types and haplotypes were considered for further
189 analyses.

190 **CHV1 sequencing**

191 Complementary DNA (cDNA) was synthesized from RNA using either the
192 GoScriptTM Reverse Transcription System (Promega Corporation, Madison, USA) or the
193 Maxima First Strand cDNA synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania)
194 following the manufacturer's protocols. PCR amplification of part of the ORF-A segment was
195 performed with the primer pairs EP713-5 and R2280 (Allemann et al. 1999) or hvep-1 and
196 EP721-4 (Bryner et al. 2012). The PCR products were sequenced by Sanger technology using
197 the same primers as mentioned above. The obtained forward and reverse sequences were
198 aligned and edited with the program CLC Main Workbench 7 (CLC bio, Aarhus, Denmark).
199 In the end, a 561 nt long consensus sequence of the viral ORF-A region was obtained from

each CHV1 isolate and used for subsequent analyses. The sequences are available in the NCBI under accession numbers MT798990-MT799084.

Population diversity analyses

The diversity of *C. parasitica* populations was estimated with the Shannon information index (*H'*) and the evenness index (*e*) which were calculated in Past 3 (Hammer, Harper and Ryan 2001) for both vc type and microsatellite data sets. Rarefaction of species richness and Shannon information index to the smallest number of isolates (31) was also performed in Past 3. The existence of linkage disequilibrium between microsatellite loci in the individual populations was tested with the index of association (I_A) and multilocus linkage disequilibrium (r_d) which were calculated with Multilocus 1.3 (Agapow and Burt 2001), with 1000 permutations. Because of low population diversity (one vc type; two and three microsatellite haplotypes), the indices *H'* and r_d were not calculated for the two North Macedonian populations.

Pairwise Mann-Whitney and Kruskall-Wallis tests were performed to test whether populations from different countries significantly differed from each other based on vc type data, as implemented in Past 3 (Hammer, Harper and Ryan 2001). Pairwise population differentiation based on microsatellite data was calculated and tested for significance in Fstat 2.9.3 (Goudet 2001). The complete data set was used to perform Principal Coordinate Analysis (PCoA), implemented in GenAlEx (Peakall and Smouse 2005, 2012). Bayesian cluster analysis was performed on complete data set using the software InStruct (Gao, Williamson, and Bustamante 2007), which does not assume Hardy-Weinberg equilibrium of populations, testing K from two to 10. Each replicate was run 200000 times followed by 100000 burn in.

CHV1 diversity

Cluster analysis of the CHV1 consensus sequences was performed in MEGA7 (Kumar, Stecher, and Tamura 2016) using a maximum likelihood algorithm with 1000 repetitions for bootstrapping. The Chinese CHV1 sequence CN280 (KT726153) was used as outgroup (Du et al. 2017). Publicly available CHV1 sequences of Italian and French subtypes I (AF082191) and F1 (NC_0011492) were used, as well as sequences of French (F2), Georgian (G), German (D), and Spanish (E) subtypes previously published by Mlinarec et al. (2018a) to ascertain the relationship with our studied CHV1 sequences.

The diversity of CHV1 sequences from different populations, as well as population differentiation (F_{ST}) of CHV1, were calculated in DnaSP6 (Rozas et al. 2017). Diversity was characterized with the number of polymorphic sites, number of haplotypes, haplotype diversity (i.e. uniqueness of a particular haplotype) and nucleotide diversity *Pi* (i.e. average number of nucleotide differences per site between two sequences). A haplotype network with CHV1 sequences was constructed with PopArt (http://popart.otago.ac.nz) using minimum spanning network model (Bandelt, Forster and Rohl 1999).

The population structure of CHV1 across the six populations was analysed using Discriminant Analysis of Principal Components (DAPC) (Jombart, Devillard and Balloux 2010) in R in order to identify clusters of closely related CHV1 sequences. One FASTA alignment including CHV1 sequences of all countries was created and clone corrected before reading it into R with the package *ape* (Paradis, Claude, and Strimmer 2004). The alignment contained a total of 98 consensus sequences with a total length of 561 nt. Identical CHV1 sequences found at different locations were included once for each location.

Correlation analysis

We used correlation analysis to assess (1) the temporal changes of vc type diversity over time, (2) the relationship between vc type diversity and the prevalence of CHV1, and (3) the relationship between fungal vc type diversity and hypovirus diversity. For the first two

analyses, population data from previous studies in Switzerland (Bryner and Rigling 2012; Cortesi et al. 1998; Hoegger et al. 2000) and unpublished data for the populations Biasca and Lattecaldo, Croatia and Slovenia (Krstin et al. 2008, 2011) and North Macedonia (Sotirovski et al. 2004, 2006) were included. These populations were chosen for comparison because they were located within a radius of 40 km from the populations sampled in the present study. For the third analysis, data from Bryner et al. (2012) were included, as this study covered a very similar geographic region as the present study with population data from Switzerland, Bosnia-Herzegovina, North Macedonia, Greece and Western Turkey. In all correlation analysis, vc type diversity was expressed as the Shannon index (*H'*). For CHV1, nucleotide diversity *Pi* (as described above) was used. All correlation analyses were done in Past 3 (Hammer, Harper and Ryan 2001) using non-parametric correlation indices.

Results

Population diversity

Both genetic markers (vc types and microsatellite) consistently showed a high *C. parasitica* population diversity in Switzerland and Croatia and a low diversity in North Macedonia (Table 1). In Swiss and Croatian populations, eight to 16 different vc types were detected, with EU-1 and EU-2 being the most common types (30-47.1%). The other vc types were represented by only one to three isolates, except EU-5 of which nine isolates were found in Contone and seven in Orselina (Supplementary Table 1). In contrast, all North Macedonian isolates belonged to the vc type EU-12, making population diversity indices *H'* and *e* non-informative (Table 1). The vast majority of EU-12 isolates from North Macedonia belonged to haplotype Cp90. EU-12 was rare in Switzerland and Croatia, with just two isolates identified in each country. One EU-12 isolate in Croatia was associated with the microsatellite

haplotype Cp90, whereas the other three EU-12 isolates (one from Croatia and two from Switzerland) were associated with haplotypes Cp3, Cp5 and Cp10, respectively.

All analysed loci were polymorphic, except locus CpE8 which was monomorphic across all populations. At loci CpG4 and I07-650 four different alleles were observed, albeit not in a single population (Supplementary Table 2). In North Macedonia two previously unobserved microsatellite alleles were identified. Several new microsatellite haplotypes, previously unreported were identified, although they occurred only once, indicating their rarity (Supplementary Table 3).

Microsatellite analyses revealed the presence of 57 different haplotypes across the six populations (Table 1). The highest haplotypic diversity was observed in the two Swiss populations (*H'* = 3.23 and 2.82), followed by the Croatian populations (*H'* = 2.26 and 2.21). With only three (Kalishte) and two (Smolare) haplotypes present, genotypic diversity of the North Macedonian populations was low (*H'* = 0.47 and 0.24, respectively). The most frequent haplotype in North Macedonia, Cp90, included 67 out of the 74 *C. parasitica* isolates, and was exclusively associated with EU-12. The two other haplotypes detected there (Cp90A and Cp90B) were closely related to Cp90, each with only one different allele at locus CPG4.

None of the haplotypes was detected in all six populations, but some haplotypes were observed in several populations, even in different countries (Supplementary Table 3). Haplotype Cp15 was observed in all three countries, albeit at different frequencies (Supplementary Table 3). Surprisingly, Cp15 haplotype isolates from different countries had various vc types: EU-12 in North Macedonia, EU-2, EU-13 and EU-40 in Croatia and EU-1, EU-2, EU-5, EU-29, and EU-42 in Switzerland. Cp33 was found 37 times, in all populations in Switzerland and Croatia and was associated with eight different vc types, five in each country. The index of association statistics rejected the hypothesis of random mating in the Croatian and Swiss populations (Table 1).

298 **Population differentiation**

299 The Kruskal-Wallis test based on vc type data showed that both North Macedonian
300 populations significantly differed from Croatian and Swiss populations (Table 2). Pairwise
301 F_{ST}-values based on microsatellite data between populations ranged from 0.0025 (Kast and
302 Orselina) to 0.7528 (Smolare and Ozalj). These differences were significant only between the
303 two North Macedonian populations and the other four populations, congruent with the
304 analysis based on vc type data (Table 2). The first two axes of the PCoA accounted for 77.2%
305 of the variation and showed that Croatian and Swiss haplotypes formed one large group,
306 which also included one (Cp15) of the four haplotypes found in North Macedonia (Fig. 1).
307 The other three North Macedonian haplotypes, including the dominant Cp90, resided outside
308 the main cluster.

309 Bayesian analysis performed with the complete microsatellite data set showed that the
310 genetic structure of our data can best be explained with K=2. This scenario indicated that
311 North Macedonian populations belonged to one cluster, while Croatian and Swiss populations
312 belonged to a different cluster (Supplementary Fig. 1).

313 **Temporal changes of vc type diversity**

314 The vc type diversity in each country was compared with previous populations studies
315 conducted in the same general study areas. In all three countries, no significant changes of vc
316 type diversity over time was observed (Fig. 2). In Switzerland, the vc type diversity has
317 remained at a similarly high level since the first population studies in 1990 (Bryner and
318 Rigling 2012; Cortesi et al. 1998; Hoegger et al. 2000 and unpublished data for the
319 populations Biasca and Lattecaldo). An overall similar pattern can be observed in Croatia
320 with only one low diversity population that was sampled in 2006 (Krstin et al. 2008). In North
321 Macedonia, vc type diversity has remained null since the first samplings in 1995 with always
322 only one vc type (EU-12) present (Sotirovski et al. 2004, 2006, 2009; Bryner et al. 2013).

323 **CHV1 prevalence and diversity**

324 Virus-infected *C. parasitica* isolates were found in all six analysed populations (Table
325 1). In both Swiss populations, hypovirulent isolates were recovered from more than 50% of
326 the cankers (57.7% in Contone and 75.0% in Orselina). Croatian and North Macedonian
327 populations showed a lower hypovirus prevalence, ranging from 25.8% in Smolare to 46.5%
328 in Kalishte. Hypovirulent isolates were observed in the most common microsatellite
329 haplotypes, as well as in the most common vc types in all populations. The prevalence of
330 CHV1 was tested for a dependency on vc type diversity, incorporating data from previous
331 studies in the three countries (Fig. 3). Although there was a large variation in CHV1
332 prevalence across the populations, no influence of vc type diversity on hypovirus prevalence
333 was observed.

334 A total of 95 CHV1 sequences were obtained, ranging from six (Smolare, Macedonia)
335 to 30 (Contone, Switzerland) per population (Table 3). Phylogenetic analysis placed all of
336 them in the same major cluster, which also included the CHV1 strain Euro 7 – a prototypic
337 sequence of the Italian subtype of CHV1 (Supplementary Fig. 2). Although North
338 Macedonian sequences tended to cluster together in the analysis, they were clearly nested
339 within the Italian subtype cluster. We detected 101 variable sites in the analysed ORF-A
340 segment in a total of 77 different CHV1 sequences. Nucleotide diversity was estimated to be
341 Pi=0.01241 (±0.00079). There was no correlation between fungal vc type diversity and
342 hypovirus nucleotide diversity (Fig. 4). The diversity of analysed CHV1 sequences was very
343 similar in all *C. parasitica* populations, regardless of the populations' vc type diversity. Most
344 of the isolates produced a unique CHV1 sequence. However, three isolates from Switzerland
345 and three isolates from Croatia shared the same CHV1 sequence. DAPC analysis clearly
346 distinguished North Macedonian from Swiss and Croatian sequences (Fig. 5). The haplotype
347 network confirmed this result, with Swiss and Croatian sequences mainly overlapping and

North Macedonian sequences forming a single (separate) cluster (Fig. 3). The prototypic CHV1-Euro7 appeared to be more related to the Swiss and Croatian CHV1 strains.

Discussion

Over the last decade, classical vc type based analyses of *C. parasitica* populations have been supplemented with microsatellite (simple sequence repeat) genotyping (Dutech et al. 2012; Ježić et al. 2012; Milgroom et al. 2008; Peters et al. 2014; Prospero and Rigling 2012). In contrast to *vic* loci, which may be under selective pressure to prevent CHV1 transmission (Milgroom and Cortesi 1999), microsatellites are selectively highly variable, thus particularly useful for investigating the invasion genetics (i.e. origin of haplotypes, gene flow, occurrence of sexual reproduction) of fungal populations. By combining the two markers, a more detailed analysis of the population structure of *C. parasitica* can be achieved. This is especially important since sweet chestnut, the host of *C. parasitica* in Europe, has a fragmented distribution range and an particular history, influenced by anthropogenic cultivation and limited gene flow between its populations (Poljak et al. 2017; Mattioni et al. 2017). Central and western European chestnut populations seem to belong to one major cluster, while the south eastern populations belong to another, with an apparent genetic barrier between them (Mattioni et al. 2017). Thus, *C. parasitica* invasion pattern might be influenced by the spatial distribution pattern of sweet chestnut.

In our study, diversity estimates of *C. parasitica* based on vc types and microsatellites gave congruent results. *Cryphonectria parasitica* populations with high vc type diversity in Switzerland and Croatia also had high microsatellite diversity and the populations with low vc type diversity in North Macedonia had a low microsatellite diversity. As expected from the genetic bases of the markers, higher diversity estimates were obtained with microsatellites than with vc type markers in all populations. Both markers revealed no differentiation

between the Swiss and Croatian populations confirming that they both belong to the central European population, which was established after the introduction of *C. parasitica* into northern Italy (Heiniger and Rigling 1994; Milgroom et al. 2008). As in previous studies conducted up to 20 years ago, this population is dominated by the vc types EU-1 and EU-2, and their two recombinants EU-5 and EU-6 (Krstin et al. 2008; Robin et al. 2000; Robin and Heiniger 2001). The high incidence of EU-1 and EU-2 in the current Swiss and Croatian populations indicates a rather stable population structure over time. This stability is remarkable, since an increase in diversity would be expected in populations which have several polymorphic *vic* loci already present and where sexual reproduction has been documented. The apparent stability of the investigated populations could be explained by lack of immigration of new genotypes (i.e. new *vic* alleles) from different gene pools, e.g. Western France or Georgia; (Dutech et al. 2012; Prospero et al. 2013), absence of significant genetic drift, frequent asexual reproduction, and sexual reproduction mainly within and among dominant vc types. Nevertheless, many additional, less frequent vc types are regularly reported in central European populations. In our study, we identified as many as 16 vc types in a single population in Switzerland (Contone) and as many as 14 in Croatia (Ozalj). Other Croatian and Swiss populations have recently been shown to have high level of vegetative type diversity (Bryner et al. 2012; Ježić et al. 2018) suggesting that the dominance of EU-1 and EU-2 might decrease in the future. A decrease of the dominant vc types will likely be associated with a further increase of vc type diversity, which could limit hypovirus transmission.

However, microsatellite analyses tend to support the overall genetic stability of *C. parasitica* populations over time both in Switzerland and in Croatia. The genetic structure of Swiss and Croatian populations in this study seems very similar i.e. the abundances of certain haplotypes are similar enough that the populations cannot be clearly distinguished from each

other, which was corroborated by PCoA. A slight increase in the number of microsatellite haplotypes in Switzerland compared to an earlier report (Prospero and Rigling 2012) might be a result of more vigorous sampling, i.e. in our study more cankers were sampled per population.

Populations in North Macedonia were clearly different than those in Switzerland and Croatia, not only by their lower diversity, but also by their dominant vc type (EU-12, rather than EU-1 or EU-2) and associated microsatellite haplotype (Cp90). This geographic population pattern is basically the same as that observed in a previous study, which analysed isolates sampled between 1993 and 2000 in Northern Italy and North Macedonia (Milgroom et al. 2008), and indicates a persistent high clonality of the *C. parasitica* population in this country. In south eastern and eastern Europe, most *C. parasitica* populations have been founded relatively recently and are dominated by vc type EU-12 (Robin and Heiniger 2001; Milgroom et al. 2008; Adamčíková et al. 2006; Jankovský et al. 2010; Radócz 2001). Our analyses found no evidence for new immigrant vc types in North Macedonia, where local populations have been composed of EU-12 since the 1990s. Interestingly, only one microsatellite haplotype, Cp15, was observed in all countries included in this study. In Switzerland and Croatia, Cp15 was associated with several different vc types, which indicated that these isolates were not clones and were formed by sexual recombination, while in North Macedonia Cp15 was associated exclusively with EU-12, suggesting its clonal spread in that country.

Taking all this in account, an invasion pattern of *C. parasitica* in Europe emerges. The central European population, which is diverse and harbours many vc types and microsatellite haplotypes, was established after the first introduction event in northern Italy in the late 1930s. Given the absence of significant geographical barriers, the central European population subsequently expanded into neighbouring regions, such as southern Switzerland,

423 eastern France, Slovenia and north-western Croatia. The source of EU-12 in south-eastern
424 Europe including North Macedonia is less evident because this vc type is rare in the central
425 European population. However, a recent genomic study suggested that the invasive EU-12
426 lineage emerged from the central European population (Stauber et al. 2020). In our study,
427 populations from Croatia and Switzerland show similar pattern of genetic diversity as other
428 central European populations, with vc types, microsatellite haplotypes and both mating types
429 already present. Sexual reproduction and probably immigration of new genotypes play major
430 roles in generating genetic diversity in these populations. South-eastern European populations
431 like North Macedonian, however, show much lower population diversity as only one vc type
432 was originally introduced and the populations appear to be mostly clonal. This spatial division
433 between central European and southern and eastern European populations in regard to vc type
434 and microsatellite haplotype diversity, has remained stable over time, as our study indicates
435 (Supplementary Table 4).

436 Hypovirulence prevalence is one of the most important factors when considering
437 chestnut blight in Europe. In genetically diverse populations with many different vc types it
438 might be problematic for CHV1 to spread efficiently. First, horizontal virus transmission
439 efficiency is significantly reduced between mycelia belonging to different vc groups, and
440 second vertical virus transmission may be hindered by the reduced sporulation of CHV1-
441 mycelia (Cortesi et al. 2001). However, in our study we did not observe such a trend. Even
442 when including data from previous studies, no correlation between vc type diversity and
443 hypovirus prevalence could be found. Vc type diversity in Europe might still be too low to act
444 as an efficient barrier for CHV1 spread within a population, unlike in the USA, where natural
445 hypovirulence spread is limited (Milgroom and Cortesi 2004). Moreover, experimental
446 studies have shown that the hypovirus can be transmitted between different vc types (Cortesi
447 et al. 2001; Liu and Milgroom 1996) and this could occur even more frequently under field

conditions than estimated from *in vitro* assays (Brusini and Robin 2013). Recently it has been demonstrated that *C. parasitica* strains with four disrupted *vic* loci were highly efficient in transmitting CHV1 to widely diverse vc types, both under laboratory conditions and in field experiments (Stauder et al. 2019). This approach is well suited for hypovirulence treatments in highly diverse population of *C. parasitica*, such as the eastern USA where biological control of the disease has failed. According to the model proposed by Milgroom and Cortesi (2004), there is a threshold of vc type diversity of about 2.0 (Shannon index), above which the hypovirus cannot successfully spread. This model threshold seems be too low, as we observed a high hypovirus prevalence (> 40%) in populations with a Shannon index higher than 2.0. Nevertheless, there is evidence that high vc type diversity, among other things, prevents successful hypovirus spread at the population level, suggested by a very low prevalence (2 - 6%) of hypoviruses in Asia (Peever et al. 1998), where vc type diversity is very high (Liu and Milgroom 2007) and failed attempts to establish CHV1 in North American populations, where vc type diversity is higher than in Europe (Milgroom and Cortesi 2004). Factors like host species and regional specificities might also contribute to the success of the CHV1 spread.

On the other hand, CHV1 could establish fairly rapidly in predominantly clonal populations in North Macedonia, as there are no genetic barriers preventing horizontal virus transmission. As late as 1998, no hypovirulent isolates were recovered in Smolare and all nearby populations on the mountain Belasica, even after extensive sampling (Sotirovski et al. 2006). Already in 2006, in the same region (Smolare) 24.2% of the sampled isolates were hypovirulent (Sotirovski et al. 2009), while in 2010 hypovirus-prevalence increased to 67% at the site Drazhevo, close to Smolare (Bryner et al. 2013). As in other European areas (Ježić et al. 2019), natural biological control of chestnut blight seems to be well established in North Macedonia, which was confirmed by the results of this study. In both investigated populations

473 (Kalishte and Smolare) inactive, healing and callused cankers were observed and hypovirulent
474 isolates were present.

475 The cluster analysis revealed, as expected, that all CHV1 isolates in this study belong
476 to the Italian (I) subtype (Gobbin et al. 2003). Since CHV1 is an RNA virus, new sequence
477 variants emerge more often from random mutations in the genome than in the DNA viruses
478 (Forterre and Prangishvili 2009; Holmes 2011). In our analysis, the CHV1 sequences showed
479 only minor differences, suggesting that all isolates share a relatively recent common ancestor.
480 This ancestor was probably introduced into Italy together with *C. parasitica* (Bryner et al.
481 2012; Mlinarec et al. 2018a) and first infected the central European population including
482 Switzerland and Croatia. Once established in the central population, a few CHV1-infected *C.*
483 *parasitica* strains migrated further to south-eastern Europe (Bryner et al. 2012). Haplotype
484 network shows that there are as many mutation steps between CHV1 strains from the two
485 North Macedonian populations as there are between North Macedonian and central European
486 (Swiss and Croatian) CHV1 populations. This pattern does not follow population
487 differentiation of *C. parasitica*, indicating that despite clonal propagation of the fungal host in
488 North Macedonia, CHV1 populations diversity arises primarily from mutations, rather than
489 migration, suggesting limited exchange of CHV1-infected *C. parasitica* strains between
490 populations.

491 All our analyses of the viruses revealed that despite the fact that all strains are closely
492 related, the North Macedonian CHV1 strains formed a distinct sub-cluster that was separated
493 from the Swiss and Croatian strains. This seems to support the hypothesis of western
494 European origin of south-eastern European CHV1 populations, with an observable bottleneck
495 and founder effect, as suggested by much closer relationship between the North Macedonian
496 CHV1 strains. This is in agreement with a previous study by Bryner et al. (2012) in which
497 central European populations from Switzerland and from Bosnia and Herzegovina formed one

cluster, while populations in south-eastern Europe and Turkey differed from this central cluster. In contrast to the fungal populations, which clearly differ in genetic diversity, there were hardly any differences in genetic diversity among the hypovirus populations. In fact, the low-diversity fungal populations in south-eastern Europe had similar levels of viral diversity as the highly diverse central populations. These results suggest a quick recovery of viral diversity from genetic bottlenecks in these recently infected fungal populations. Strong population growth in the clonal host populations combined with a high mutation rate, typically for RNA viruses, could explain the rapid increase in viral diversity after a genetic bottleneck.

Conclusions

The use of genetic markers for both *C. parasitica* and associated hypovirus allowed us to gain a deeper insight into the temporal and spatial population structure across an invasive range of chestnut blight in Europe. Our study revealed a stable gradient of genetic diversity of the pathogen over time from the more diverse central European population to the more clonal population in south-eastern Europe. Hypovirulence established itself throughout the invasive range and was little influenced by the diversity of vegetative compatibility types. Genetic diversity of the hypovirus reached similar levels in all populations regardless of the age and diversity of the fungal populations. This finding indicates a fast recovery of the virus diversity after a genetic bottleneck in newly infected host populations.

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Literature Cited

Agapow, P.-M., and Burt, A. 2001. Indices of multilocus linkage disequilibrium. Mol. Ecol. Notes. 1:101–102.

Allemann, C., Hoegger, P., Heiniger, U., and Rigling, D. 1999. Genetic variation of *Cryphonectria hypoviruses* (CHV1) in Europe, assessed using restriction fragment length polymorphism (RFLP) markers. Mol. Ecol. 8:843–854.

Anagnostakis, S. L. 1987. Chestnut Blight: The Classical Problem of an Introduced Pathogen. Mycologia. 79:23.

Anagnostakis, S. L. 1988. *Cryphonectria parasitica*, Cause of Chestnut Blight. In *Advances in Plant Pathology*, , p. 123–136.

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R., and Daszak, P. 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends Ecol. Evol. 19:535–544.

Aukema, J. E., Leung, B., Kovacs, K., Chivers, C., Britton, K. O., Englin, J., Frankel, S. J., Haight, R. G., Holmes, T. P., Liebhold, A. M., McCullough, D. G., and von Holle, B. 2011. Economic Impacts of Non-Native Forest Insects in the Continental United States ed. Brian Gratwicke. PLoS One. 6:e24587.

Bandelt, H. J., Forster, P., and Rohl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16:37–48.

Bissegger, M., Rigling, D., and Heiniger, U. 1997. Population Structure and Disease

548 Development of *Cryphonectria parasitica* in European Chestnut Forests in the Presence of
549 Natural Hypovirulence. Phytopathology. 87:50–59.
550 Breuillin, F., Dutech, C., and Robin, C. 2006. Genetic diversity of the Chestnut blight fungus
551 *Cryphonectria parasitica* in four French populations assessed by microsatellite markers.
552 Mycol. Res. 110:288–296.
553 Brusini, J., and Robin, C. 2013. Mycovirus transmission revisited by in situ pairings of
554 vegetatively incompatible isolates of *Cryphonectria parasitica*. J. Virol. Methods. 187:435–
555 442.
556 Bryner, S. F., and Rigling, D. 2012. Hypovirus Virulence and Vegetative Incompatibility in
557 Populations of the Chestnut Blight Fungus. Phytopathology. 102:1161–1167.
558 Bryner, S. F., Rigling, D., and Brunner, P. C. 2012. Invasion history and demographic pattern
559 of *Cryphonectria hypovirus 1* across European populations of the chestnut blight fungus.
560 Ecol. Evol. 2:3227–3241.
561 Bryner, S. F., Sotirovski, K., Akilli, S., Risteski, M., Perlerou, C., and Rigling, D. 2013.
562 Informative value of canker morphology on the presence or absence of virus infection in
563 chestnut blight cankers. For. Pathol. 43:496–504.
564 Cortesi, P., McCulloch, C. E., Song, H., Lin, H., and Milgroom, M. G. 2001. Genetic control
565 of horizontal virus transmission in the chestnut blight fungus, *Cryphonectria parasitica*.
566 Genetics. 159:107–118.
567 Cortesi, P., and Milgroom, M. G. 1998. Genetics of Vegetative Incompatibility in
568 *Cryphonectria parasitica*. Appl. Environ. Microbiol. 64:2988–2994.
569 Cortesi, P., Rigling, D., and Heiniger, U. 1998. Comparison of vegetative compatibility types
570 in Italian and Swiss subpopulations of *Cryphonectria parasitica*. For. Pathol. 28:167–176.
571 Du, Y., Lin, Y., Zhou, X., Wang, K., Fang, S., and Deng, Q. 2017. Full-length sequence and
572 genome analysis of CHV1-CN280, a North China isolate of *Cryphonectria hypovirus 1*. Arch.

573 Virol. 162:1811–1818.
574 Dutech, C., Barrès, B., Bridier, J., Robin, C., Milgroom, M. G., and Ravigné, V. 2012. The
575 chestnut blight fungus world tour: successive introduction events from diverse origins in an
576 invasive plant fungal pathogen. Mol. Ecol. 21:3931–3946.
577 Dutech, C., Fabreguettes, O., Capdevielle, X., and Robin, C. 2010. Multiple introductions of
578 divergent genetic lineages in an invasive fungal pathogen, *Cryphonectria parasitica*, in
579 France. Heredity (Edinb). 105:220–228.
580 Feau, N., Dutech, C., Brusini, J., Rigling, D., and Robin, C. 2014. Multiple introductions and
581 recombination in *Cryphonectria hypovirus 1*: perspective for a sustainable biological control
582 of chestnut blight. Evol. Appl. 7:580–596.
583 Forterre, P., and Prangishvili, D. 2009. The origin of viruses. Res. Microbiol. 160:466–472.
584 Gao, H., Williamson, S., and Bustamante, C. D. 2007. A Markov Chain Monte Carlo
585 Approach for Joint Inference of Population Structure and Inbreeding Rates From Multilocus
586 Genotype Data. Genetics. 176:1635–1651.
587 Gobbin, D., Hoegger, P. ., Heiniger, U., and Rigling, D. 2003. Sequence variation and
588 evolution of *Cryphonectria hypovirus 1* (CHV-1) in Europe. Virus Res. 97:39–46.
589 Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices
590 Version 2.9.3.
591 Gross, A., Hosoya, T., and Queloz, V. 2014. Population structure of the invasive forest
592 pathogen *Hymenoscyphus pseudoalbidus*. Mol. Ecol. 23:2943–2960.
593 Hammer, Ø., Harper, D. A. T., and Ryan, P. D. 2001. Past: paleontological statistics software
594 package for education and data analysis. Palaeontol. Electron. 4:9pp.
595 Heiniger, U., and Rigling, D. 1994. Biological Control of Chestnut Blight in Europe. Annu.
596 Rev. Phytopathol. 32:581–599.
597 Hillman, B. I., and Suzuki, N. 2004. Viruses of the Chestnut Blight Fungus, *Cryphonectria*

598 *parasitica*. In *Advances in Virus Research*, Advances in Virus Research, Academic Press, p.
599 423–472.
600 Hoegger, P. J., Rigling, D., Holdenrieder, O., and Heiniger, U. 2000. Genetic structure of
601 newly established populations of *Cryphonectria parasitica*. Mycol. Res. 104:1108–1116.
602 Holmes, E. C. 2011. What Does Virus Evolution Tell Us about Virus Origins? J. Virol.
603 85:5247–5251.
604 Ježić, M., Kolp, M., Prospero, S., Sotirovski, K., Double, M., Rigling, D., Risteski, M., Karin-
605 Kujundžić, V., Idžojtić, M., Poljak, I., and Ćurković-Perica, M. 2019. Diversity of
606 *Cryphonectria parasitica* in callused chestnut blight cankers on European and American
607 chestnut. For. Pathol. 49:e12566.
608 Ježić, M., Krstin, L., Rigling, D., and Ćurkovć-Perica, M. 2012. High diversity in populations
609 of the introduced plant pathogen, *Cryphonectria parasitica*, due to encounters between
610 genetically divergent genotypes. Mol. Ecol. 21:87–99.
611 Ježić, M., Mlinarec, J., Vuković, R., Katanić, Z., Krstin, L., Nuskern, L., Poljak, I., Idžojtić,
612 M., Tkalec, M., and Ćurković-Perica, M. 2018. Changes in *Cryphonectria parasitica*
613 populations affect natural biological control of chestnut blight. Phytopathology. 108:870–877.
614 Jombart, T., Devillard, S., and Balloux, F. 2010. Discriminant analysis of principal
615 components: a new method for the analysis of genetically structured populations. BMC
616 Genet. 11:94.
617 Krstin, L., Katanić, Z., Repar, J., Ježić, M., Kobaš, A., and Ćurković-Perica, M. 2020. Genetic
618 Diversity of *Cryphonectria hypovirus 1*, a Biocontrol Agent of Chestnut Blight, in Croatia
619 and Slovenia. Microb. Ecol. 79:148–163.
620 Krstin, L., Novak-Agbaba, S., Rigling, D., and Ćurković-Perica, M. 2011. Diversity of
621 vegetative compatibility types and mating types of *Cryphonectria parasitica* in Slovenia and
622 occurrence of associated *Cryphonectria hypovirus 1*. Plant Pathol. 60:752–761.

623 Krstin, L., Novak-Agbaba, S., Rigling, D., Krajačić, M., and Ćurković-Perica, M. 2008.
624 Chestnut blight fungus in Croatia: diversity of vegetative compatibility types, mating types
625 and genetic variability of associated *Cryphonectria hypovirus 1*. Plant Pathol. 57:1086–1096.
626 Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics
627 Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33:1870–1874.
628 Liebhold, A. M., Brockerhoff, E. G., Garrett, L. J., Parke, J. L., and Britton, K. O. 2012. Live
629 plant imports: the major pathway for forest insect and pathogen invasions of the US. Front.
630 Ecol. Environ. 10:135–143.
631 Liu, Y.-C., and Milgroom, M. G. 2007. High diversity of vegetative compatibility types in
632 *Cryphonectria parasitica* in Japan and China. Mycologia. 99:279–284.
633 Liu, Y., and Milgroom, M. 1996. Correlation between hypovirus transmission and the number
634 of vegetative incompatibility (*vic*) genes different among isolates from a natural population of
635 *Cryphonectria parasitica*. Phytopathology. 86:79–86.
636 Mattioni, C., Martin, M. A., Chiocchini, F., Cherubini, M., Gaudet, M., Pollegioni, P.,
637 Velichok, I., Jarman, R., Chambers, F. M., Paule, L., Damina, V. L., Crainic, G. C. and
638 Villani, F. 2017. Landscape genetics structure of European sweet chestnut (*Castanea sativa*
639 Mill): indications for conservation priorities. Tree Genet. Genomes. 13:39.
640 Milgroom, M. G., and Cortesi, P. 1999. Analysis of population structure of the chestnut blight
641 fungus based on vegetative incompatibility genotypes. Proc. Natl. Acad. Sci. U. S. A.
642 96:10518–10523.
643 Milgroom, M. G., and Cortesi, P. 2004. Biological control of chestnut blight with
644 hypovirulence: A Critical Analysis. Annu. Rev. Phytopathol. 42:311–338.
645 Milgroom, M. G., Sotirovski, K., Spica, D., Davis, J. E., Brewer, M. T., Milev, M., and
646 Cortesi, P. 2008. Clonal population structure of the chestnut blight fungus in expanding
647 ranges in southeastern Europe. Mol. Ecol. 17:4446–4458.

Mlinarec, J., Ježić, M., Ćosić, J., and Ćurković-Perica, M. 2018a. Multilocus PCR assay reveals high diversity of vegetative compatibility types in populations of *Cryphonectria parasitica* in Croatia. Plant Pathol. 67:741–749.

Mlinarec, Jelena, Nuskern, L., Ježić, M., Rigling, D., and Ćurković-Perica, M. 2018b. Molecular evolution and invasion pattern of *Cryphonectria hypovirus 1* in Europe: Mutation rate, and selection pressure differ between genome domains. Virology. 514:156–164.

Paradis, E., Claude, J., and Strimmer, K. 2004. APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics. 20:289–290.

Peakall, R., and Smouse, P. E. 2005. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes. 6:288–295.

Peakall, R., and Smouse, P. E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics. 28:2537–2539.

Peever, T. L., Liu, Y.-C. Y.-C., Cortesi, P., and Milgroom, M. G. 2000. Variation in Tolerance and Virulence in the Chestnut Blight Fungus-Hypovirus Interaction. Appl. Environ. Microbiol. 66:4863–4869.

Peever, T. L., Liu, Y. C., Wang, K., Hillman, B. I., Foglia, R., and Milgroom, M. G. 1998. Incidence and Diversity of Double-Stranded RNAs Occurring in the Chestnut Blight Fungus, *Cryphonectria parasitica*, in China and Japan. Phytopathology. 88:811–817.

Peters, F. S., Bußkamp, J., Prospero, S., Rigling, D., and Metzler, B. 2014. Genetic diversification of the chestnut blight fungus *Cryphonectria parasitica* and its associated hypovirus in Germany. Fungal Biol. 118:193–210.

Poljak, I., Idžojtić, M., Šatović, Z., Ježić, M., Ćurković-Perica, M., Simovski, B., Acevski, J, and Liber, Z. 2017. Genetic diversity of the sweet chestnut (*Castanea sativa* Mill.) in Central Europe and the western part of the Balkan Peninsula and evidence of marron genotype introgression into wild populations. Tree Genet. Genomes. 13:18.

673 Prospero, S., Lutz, A., Tavadze, B., Supatashvili, A., and Rigling, D. 2013. Discovery of a
674 new gene pool and a high genetic diversity of the chestnut blight fungus *Cryphonectria*
675 *parasitica* in Caucasian Georgia. Infect. Genet. Evol. 20:131–139.
676 Prospero, S., and Rigling, D. 2012. Invasion Genetics of the Chestnut Blight Fungus
677 *Cryphonectria parasitica* in Switzerland. Phytopathology. 102:73–82.
678 Radócz, L. 2001. Study of subpopulations of the chestnut blight (*Cryphonectria parasitica*)
679 fungus in the Carpathian basin. For. Snow Landsc. Res. 76:368–372.
680 Rigling, D., Borst, N., Cornejo, C., Supatashvili, A., and Prospero, S. 2018. Genetic and
681 Phenotypic Characterization of *Cryphonectria hypovirus 1* from Eurasian Georgia. Viruses.
682 10:687.
683 Rigling, D., and Prospero, S. 2018. *Cryphonectria parasitica*, the causal agent of chestnut
684 blight: invasion history, population biology and disease control. Mol. Plant Pathol. 19:7–20.
685 Risteski, M., Milev, M., Rigling, D., Milgroom, M. G., Bryner, S. F., and Sotirovski, K. 2013.
686 Distribution of chestnut blight and diversity of *Cryphonectria parasitica* in chestnut forests in
687 Bulgaria. For. Pathol. 43:437–443.
688 Robin, C., Anziani, C., and Cortesi, P. 2000. Relationship Between Biological Control,
689 Incidence of Hypovirulence, and Diversity of Vegetative Compatibility Types of
690 *Cryphonectria parasitica* in France. Phytopathology. 90:730–737.
691 Robin, C., and Heiniger, U. 2001. Chestnut blight in Europe: diversity of *Cryphonectria*
692 *parasitica*, hypovirulence and biocontrol. For. Snow Landsc. Res. 76:361–367.
693 Robin, C., Lanz, S., Soutrenon, A., and Rigling, D. 2010. Dominance of natural over released
694 biological control agents of the chestnut blight fungus *Cryphonectria parasitica* in south-
695 eastern France is associated with fitness-related traits. Biol. Control. 53:55–61.
696 Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-
697 Onsins, S. E., et al. 2017. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data

Sets. Mol. Biol. Evol. 34:3299–3302.

Santini, A., Ghelardini, L., De Pace, C., Desprez-Loustau, M. L., Capretti, P., Chandelier, A., Cech, T, Chira, D., Diamandis, S, Gaitniekis, T, Hantula, H, Holdenrieder, O., Jankovski, L., Jung, T., Jurc, D., Kirisits, T, Kunca, A, Lygis, V., Malecka, M., Marcais, B., Schmitz, S., Schumacher, J., Solheim, H., Solla, A., Szabo, I., Tsopela, P., Vannini, A., Vettaino, A. M., Webber, J., Woodward, S, and Stenlid, J. 2013. Biogeographical patterns and determinants of invasion by forest pathogens in Europe. New Phytol. 197:238–250.

Short, D. P. G., Double, M., Nuss, D. L., Stauder, C. M., MacDonald, W., and Kasson, M. T. 2015. Multilocus PCR Assays Elucidate Vegetative Incompatibility Gene Profiles of *Cryphonectria parasitica* in the United States Appl. Environ. Microbiol. 81:5736–5742.

Sotirovski, K., Milgroom, M. G., Rigling, D., and Heiniger, U. 2006. Occurrence of *Cryphonectria hypovirus 1* in the chestnut blight fungus in Macedonia. For. Pathol. 36:136–143.

Sotirovski, K., Papazova-Anakieva, I., Grünwald, N., and Milgroom, M. 2004. Low diversity of vegetative compatibility types and mating type of *Cryphonectria parasitica* in the southern Balkans. Plant Pathol. 53:325–333.

Stauber, L., Prospero, S., and Croll, D. 2020. Emergence and diversification of a highly invasive chestnut pathogen lineage across south-eastern Europe. Preprint from bioRxiv. Doi: 10.1101/2020.02.15.950170.

Stauder, C. M., Nuss, D. L., Zhang, D. X., Double, M. L., MacDonald, W. L., Metheny, A. M., et al. 2019. Enhanced hypovirus transmission by engineered super donor strains of the chestnut blight fungus, *Cryphonectria parasitica*, into a natural population of strains exhibiting diverse vegetative compatibility genotypes. Virology. 528:1–6.

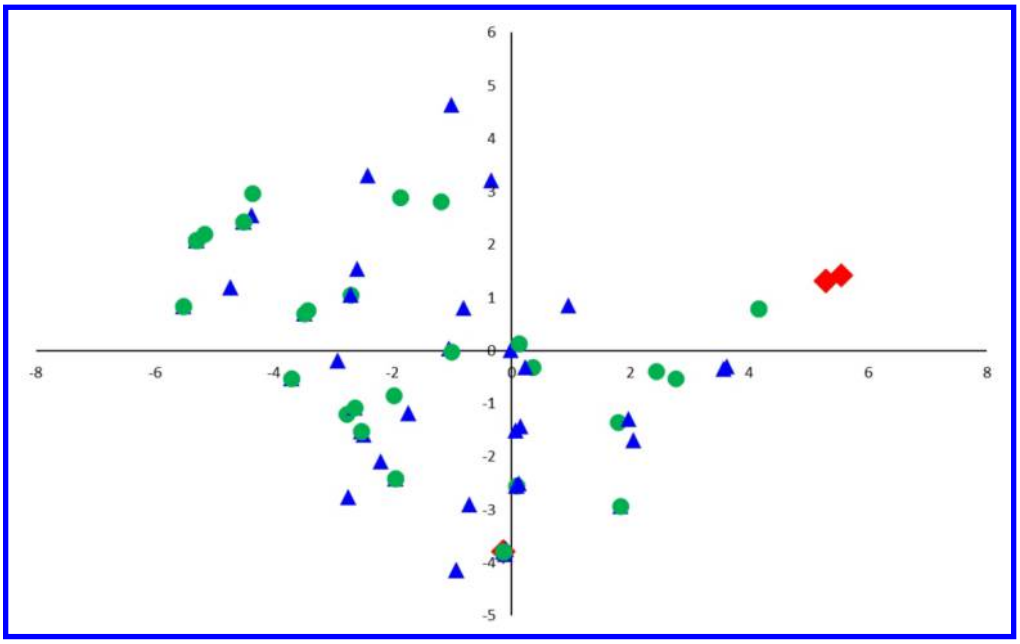


Fig.1 Principal coordinate analysis (PCoA) of Swiss (blue triangles), Croatian (green circles) and North Macedonian (red diamonds) microsatellite haplotypes of *Cryphonectria parasitica*.

195x121mm (300 x 300 DPI)

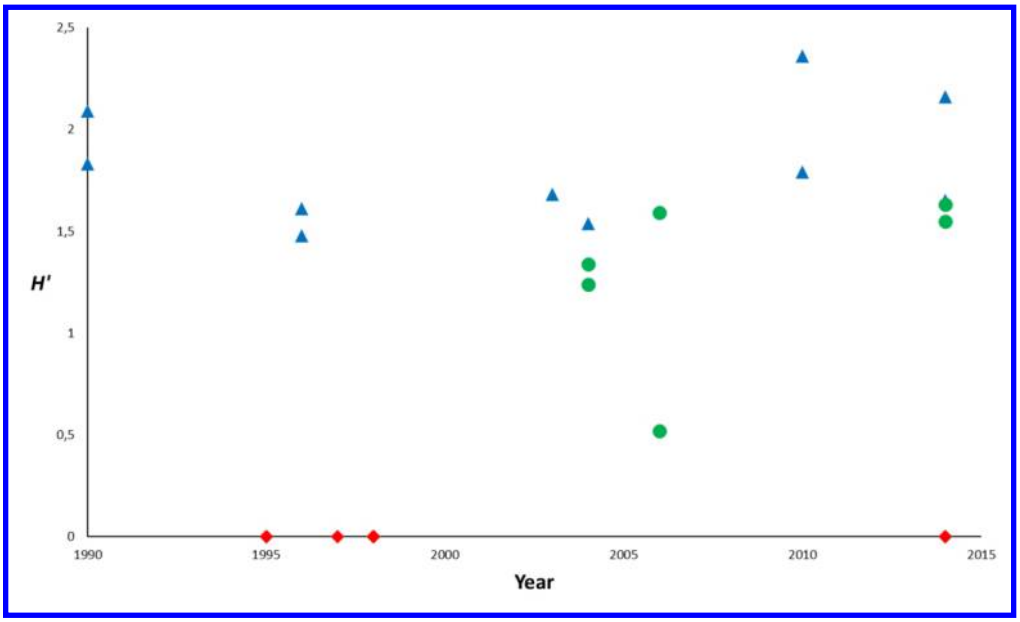


Fig. 2 Diversity of vegetative compatibility (vc) types in Swiss (blue triangles), Croatian (green circles) and North Macedonian (red diamonds) *Cryphonectria parasitica* populations in different sampling years. Diversity is expressed as the Shannon index (*H'*). There was no indication for a significant change of vc type diversity over time in all populations. Correlation analysis for Switzerland: Spearman ρ = 0.541, p = 0.219; Croatia & Slovenia: Spearman ρ = 0.358, p = 0.460; North Macedonia: Spearman ρ = 0, p = 1.

390x234mm (300 x 300 DPI)

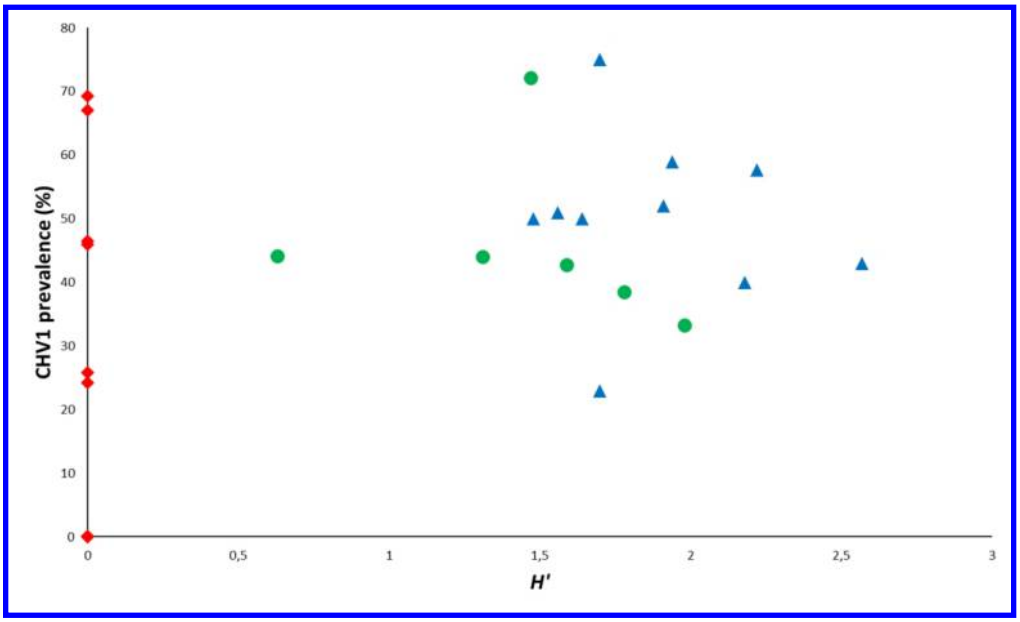


Fig. 3 Hypovirus prevalence in Swiss (blue triangles), Croatian (green circles) and North Macedonian (red diamonds) as a function of fungal vc type diversity in European *Cryphonectria parasitica* populations. Fungal vc type diversity is expressed as the Shannon index (*H'*). Correlation analysis: Spearman ρ = 0.0176, p = 0.945.

390x234mm (300 x 300 DPI)

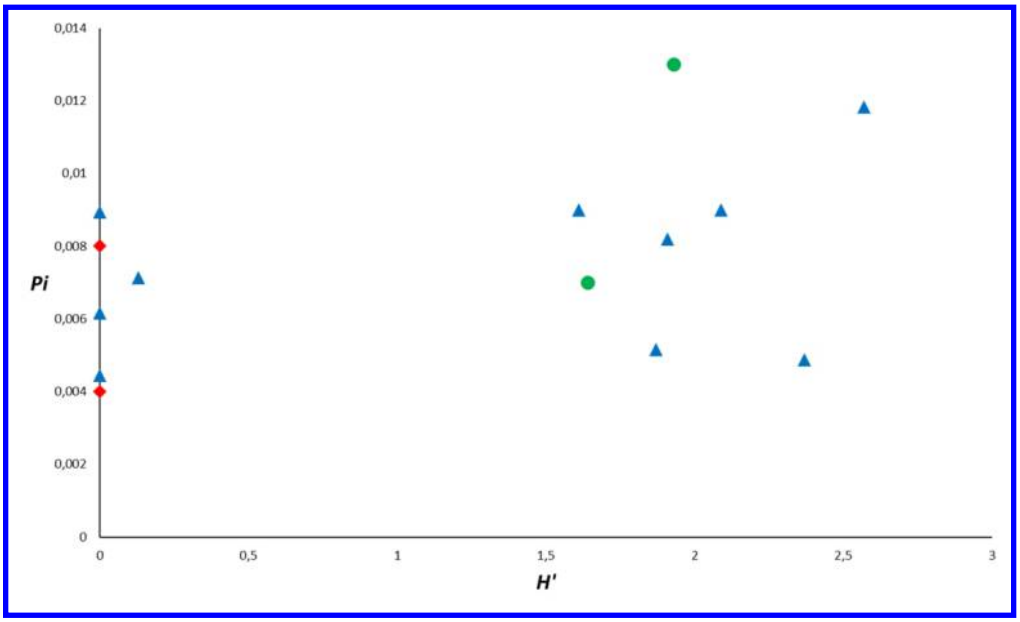


Fig. 4 Hypovirus nucleotide diversity in Swiss (blue triangles), Croatian (green circles) and North Macedonian (red diamonds) as a function of fungal vc type diversity in European *Cryphonectria parasitica* populations. Fungal vc type diversity is expressed as the Shannon index (*H'*) and virus nucleotide diversity as the average number of nucleotide differences per site between two sequences (*Pi*). Correlation analysis: Spearman ρ = 0.4501, p = 0.1064.

390x234mm (300 x 300 DPI)

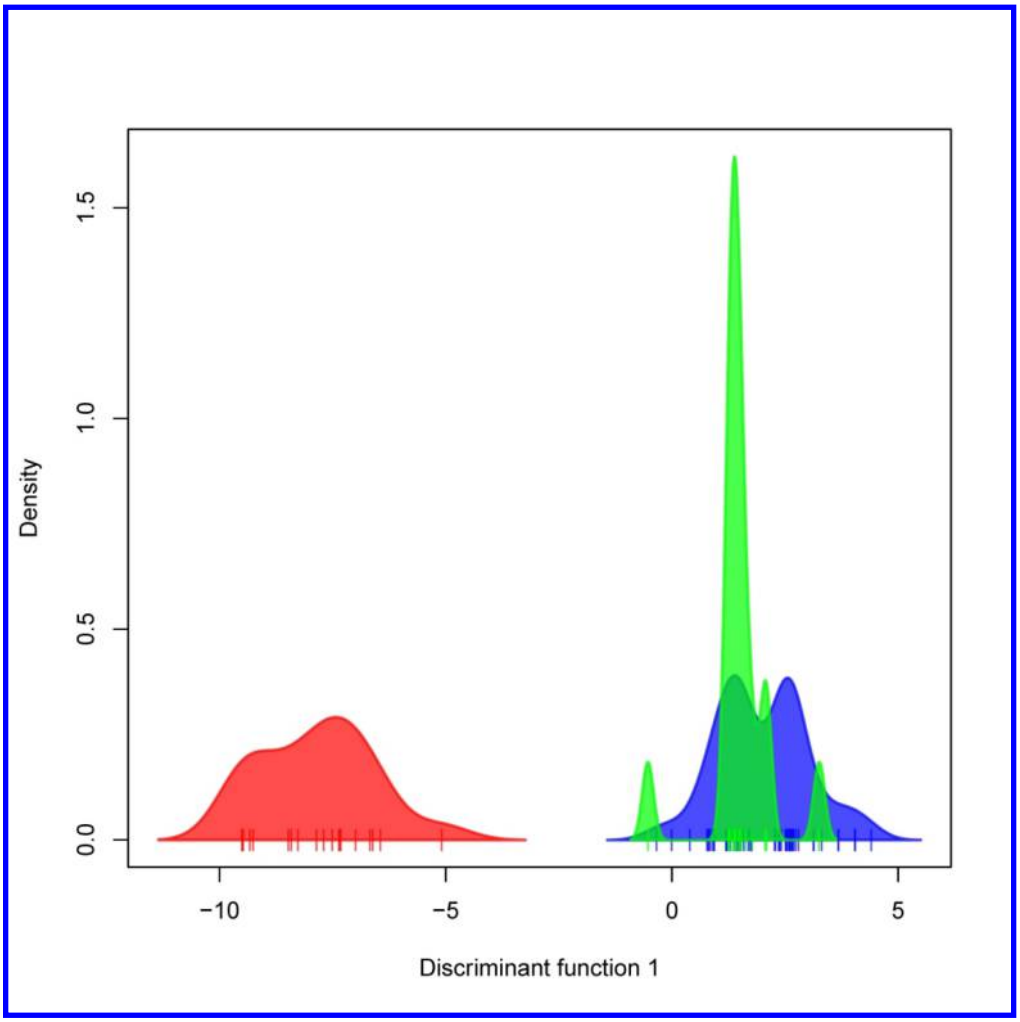


Fig. 5 Discriminant analysis of principal components (DAPC) for all *Cryphonectria hypovirus 1* (CHV1) haplotypes across the study area. Haplotypes from different countries are indicated by different colours: Switzerland – Blue, Croatia – Green, North Macedonia – Red.

177x177mm (300 x 300 DPI)

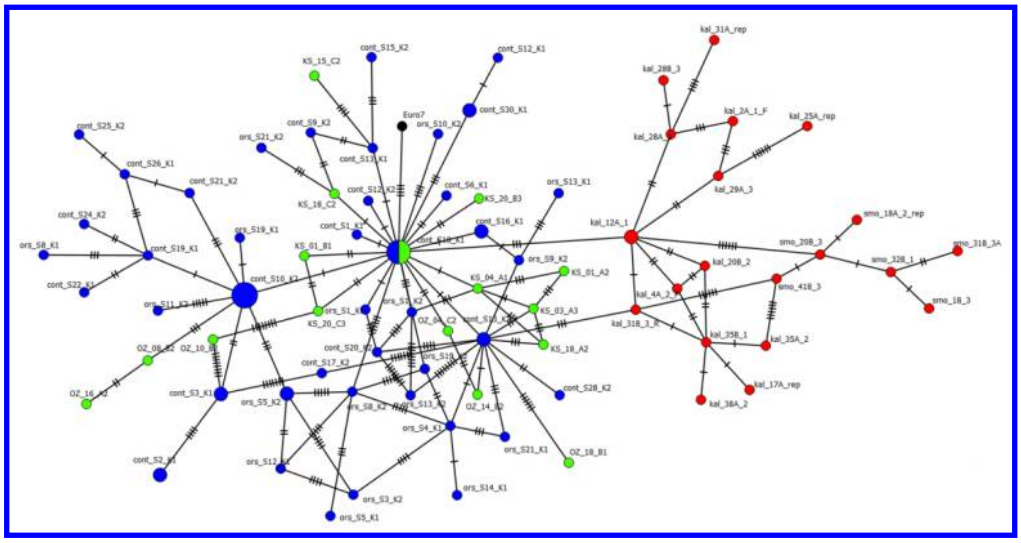


Fig. 6 Haplotype network created in Popart using 561 bp long *Cryphonectria hypovirus 1* (CHV1) sequences from ORF-A region of the genome. Size of the circle correspond to the number of isolates sharing the same consensus sequence. Isolates originating from Switzerland are represented with blue, from Croatia with green and from North Macedonia with red colour. The bars indicate number of single nucleotide mutations separating two sequences.

793x413mm (600 x 600 DPI)

Table 1. Summary statistics of the diversity of Swiss (Contone, Orselina), Croatian (Kast, Ozalj) and North Macedonian (Kalishte, Smolare) *Cryphonectria parasitica* populations based on vc type and microsatellite data. For Shannon diversity index (*H'*) and evenesss index (*e*), the 95% confidence interval is given in parentheses. Statistically significant ($p < 0.05$) modified index of association r_d is indicated with an asterisk. Because of the presence of only one vc type and 2-3 microsatellite haplotypes, the indices *H'* and r_d could not be calculated (n.a.) for the two North Macedonian populations.

	Switzerland		Croatia		North Macedonia	
Populations	Contone	Orselina	Kast	Ozalj	Kalishte	Smolare
C. parasitica isolates, N (%)[1]	52 (86.7)	52 (86.7)	39 (65.0)	36 (60.0)	43 (53.8)	31 (37.8)
CHV1-infected cankers, N (%)[2]	30 (57.7)	39 (75.0)	15 (38.5)	12 (33.3)	20 (46.5)	8 (25.8)
1) Vc type diversity						
Vc types, N	16	11	8	14	1	1
Most common vc type (%)[3]	EU-2 (30)	EU-2 (38.5)	EU-1 (47.1)	EU-2 (36.1)	EU-12 (100)	EU-12 (100)
Shannon diversity index, *H'* (95% C.I.)	2.22 (1.79-2.37)	1.70 (1.32-1.92)	1.78 (1.39-1.96)	1.98 (1.40-2.20)	n.a.	n.a.
Evenness, *e* (95% C.I.)	0.58 (0.51-0.73)	0.50 (0.44-0.64)	0.66 (0.49-0.79)	0.51 (47-0.71)	n.a.	n.a.
Richness rarefaction for (n=31)	11.87	8.11	7.71	12.45	1	1
Shannon rarefaction (n=31)	2.09	1.61	1.64	1.93	0	0
2) Microsatellite diversity						
Haplotypes, N	32	23	12	16	3	2

Most frequent haplotype (%)	Cp7 (14)	Cp33 (15.4)	Cp33 (41.2)	Cp33 (30.6)	Cp90 (86)	Cp90 (93.5)
Shannon diversity index, *H'* (95% C.I.)	3.23 (2.9-3.5)	2.82 (2.6-3.1)	2.26 (2.0-2.5)	2.21 (1.9-2.5)	0.47 (0.3-0.7)	0.24 (0.1-0.4)
Evenness, *e* (95% C.I.)	0.93 (0.9-1.0)	0.90 (08-0.9)	0.91 (0.8-1.0)	0.80 (0.7-0.9)	0.43 (0.2-0.6)	0.35 (0.1-0.6)
Richness rarefaction for (n=31)	22.16	18.34	13.66	15.41	2.72	2
Shannon rarefaction (N=31)	2.93	2.67	2.13	2.34	0.45	0.24
Modified index of association, r_d	0.127*	0.031*	0.194*	0.146*	n.a.	n.a.

[1]Number of bark cankers from which at least one *C. parasitica* isolate could be recovered.

[2]Number of bark cankers from which at least one hypovirus-infected *C. parasitica* isolates could be recovered (percentage (%) refers to the total number of cankers yielding *C. parasitica*).

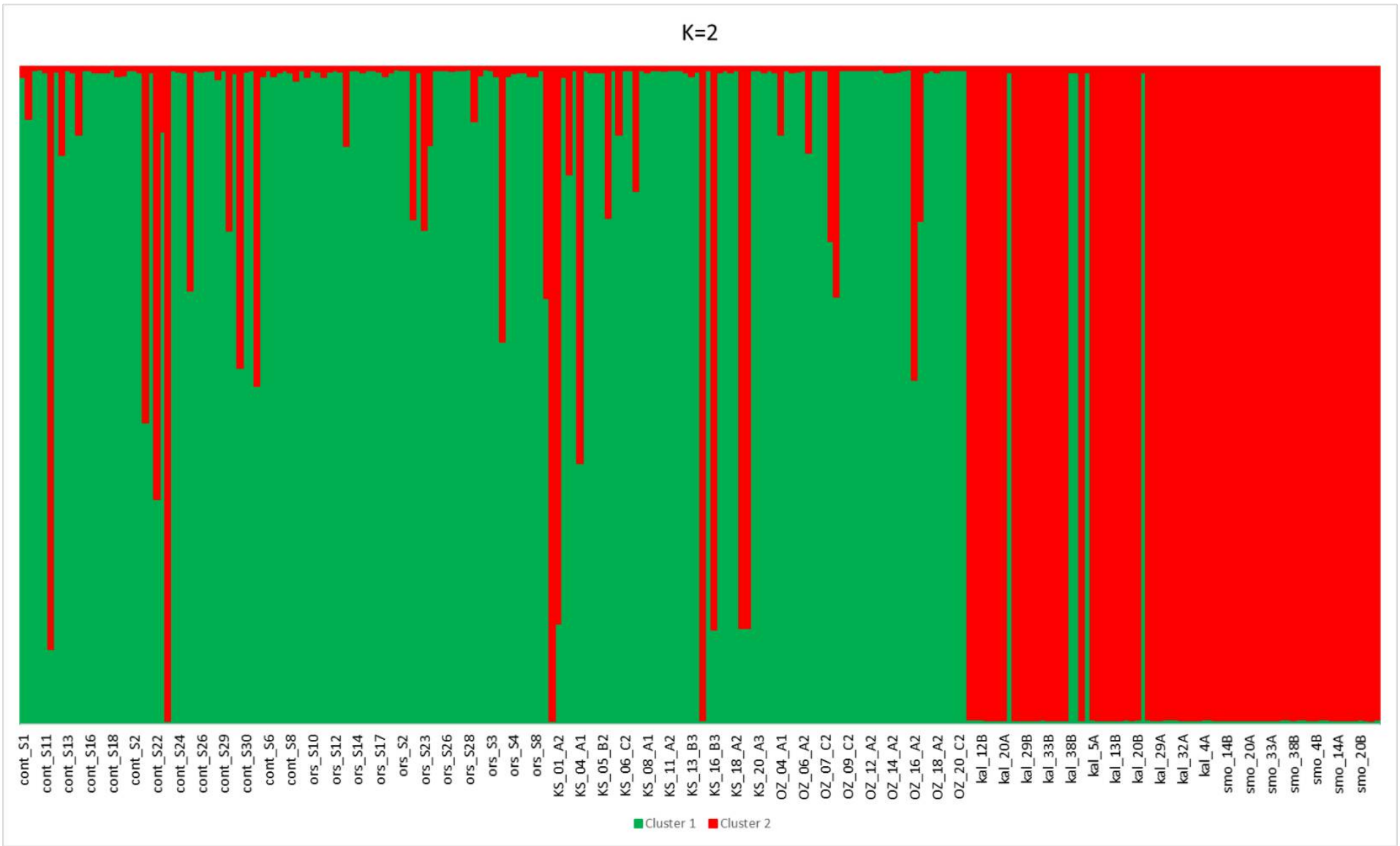
[3]Frequencies of all vc types are given in Supplementary Table 1.

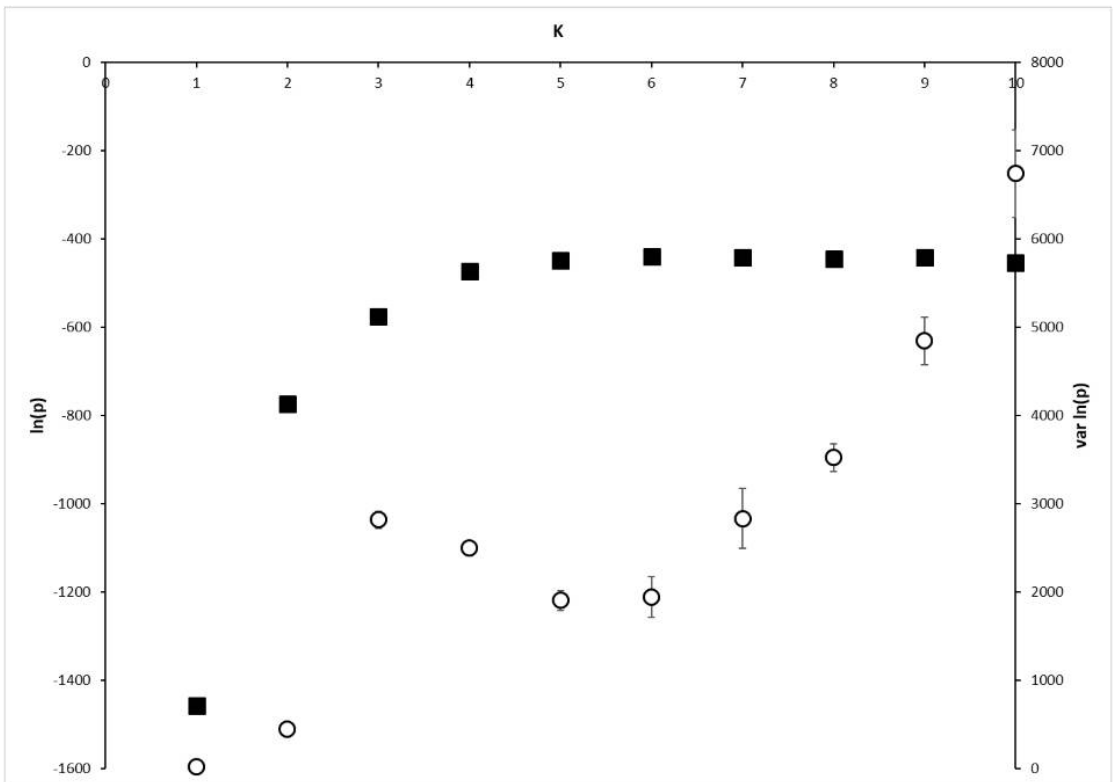
Table 2. Mann-Whitney pairwise analysis of differences between *Cryphonectria parasitica* populations based on vc type data (below the diagonal) and pairwise F_{ST}-values based on microsatellite data (above the diagonal). Significant p-values are indicated with an asterisk (*).

	Switzerland		Croatia		North Macedonia	
Population	Contone	Orselina	Kast	Ozalj	Kalishte	Smolare
Contone	-	0.028	0.025	0.05	0.546*	0.633*
Orselina	0.084	-	0.002	0.01	0.638*	0.727*
Kast	0.173	0.715	-	0.013	0.567*	0.661*
Ozalj	0.964	0.289	0.440	-	0.664*	0.753*
Kalishte	4.3^{-05}*	0.002*	0.016*	0.0002*	-	0.08
Smolare	4.3^{-05}*	0.002*	0.016*	0.0002*	0.979	-

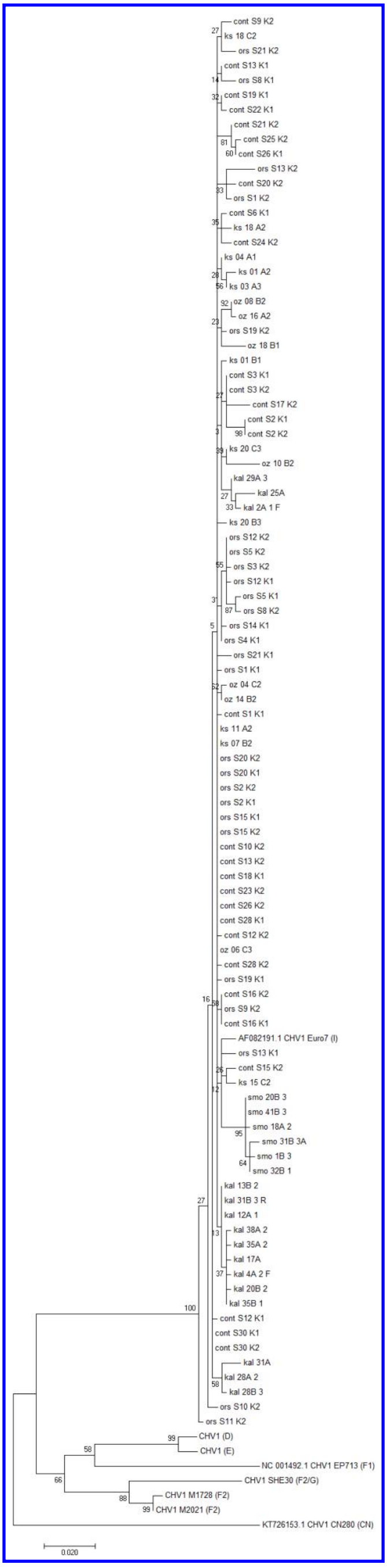
Table 3. *Cryphonectria hypovirus 1* (CHV1) diversity in the six *Cryphonectria parasitica* populations analysed in this study. All sequences of the ORF-A were 561 bp in length. Number of analysed sequences per population, number of CHV1 sequences, single nucleotide polymorphisms (SNPs), nucleotide diversity (*Pi*) and haplotype diversity (*Hd*) are given for each population.

	Switzerland		Croatia		North Macedonia	
Population	Contone	Orselina	Kast	Ozalj	Kalishte	Smolare
Sequences (N)	30	26	11	7	15	6
Haplotypes (N)	22	20	10	7	13	5
SNPs (N)	35	40	17	21	21	5
Pi	0.009	0.009	0.007	0.013	0.008	0.004
Hd	0.968	0.950	0.981	1.0	0.971	0.933





Supplementary figure 1. Bayesian cluster analysis of *Cryphonectria parasitica* populations with InStruct. Posterior probability and variance for K=2 to 10 with standard deviation given after 15 iterations of each K tested, graph for complete data set. Individuals from Switzerland and Croatia mostly affiliated with one cluster (green), while North Macedonian isolates affiliated with the second cluster (red).



Supplementary figure 2. Cluster analysis of *Cryphonectria hypovirus 1* (CHV1) sequences obtained from Croatia, Switzerland and North Macedonia. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) in MEGA7 with bootstrap values obtained after 1000 iterations.

Supplementary table 1. Absolut numbers (N) and frequencies (n_i) of vc types across four investigated populations from Swizterland and Croatia. In North Macedonia EU-12 was the only vc type observed, thus populations Kalishte and Smolare are excluded.

	Switzerland				Croatia			
	Contone		Orselina		Kašt		Ozalj	
EU-type	N	n_i	N	n_i	N	n_i	N	n_i
EU-1	8	0.16	15	0.29	16	0.42	10	0.28
EU-2	15	0.3	20	0.38	9	0.24	13	0.36
EU-3	2	0.04	1	0.02			1	0.03
EU-5	9	0.18	7	0.13	2	0.05	2	0.06
EU-6	2	0.04	1	0.02				
EU-7			1	0.02				
EU-8	1	0.02	1	0.02				
EU-9	1	0.02						
EU-11							1	0.03
EU-12	2	0.04			1	0.03	1	0.03
EU-13	3	0.06			2	0.05	1	0.03
EU-14			2	0.04			1	0.03
EU-15			1	0.02				
EU-16	1	0.02						
EU-17	1	0.02			3	0.08	1	0.03
EU-18	1	0.02						
EU-21							1	0.03
EU-23	1	0.02	1	0.02				
EU-28							1	0.03
EU-29	1	0.02						
EU-30							1	0.03
EU-31	1	0.02						
EU-33					2	0.05		
EU-39							1	0.03
EU-40					3	0.08	1	0.03
EU-42	1	0.02						
EU-47			2	0.04				
Σ	50		52		38		36	

Supplementary table 2. Number of alleles detected (*Na*), gene diversity (H_T), genetic differentiation (G_{ST}) and allelic frequencies of ten microsatellite loci of *C. parasitica* populations investigated in this research. New alleles detected in this research are in bold and indicated with asterisk. Alleles common in Switzerland and Croatia, but rare in North Macedonia or vice versa are in bold marked with †. Private alleles are in bold and indicated with *****.

					Switzerland		Croatia		North Macedonia	
Locus	*Na*	Allele	H_T	G_{ST}	Contone	Orselina	Kast	Ozalj	Kalishte	Smolare
CpE1	2	130	0.46	0.31	0,54	0,50	0,46	0,31	1,00	1,00
		148			0,46	0,50	0,54	0,69	0,00	0,00
CpE5	3	252	0.52	0.57	**0,82†**	**0,89†**	**0,79†**	**0,86†**	0,12	0,00
		255			0,10	0,07	0,15	0,09	**0,88†**	**1,00†**
		260			0,08	0,04	0,05	0,06	0,00	0,00
CpG14	2	256	0.38	0.14	0,52	0,72	0,74	0,60	0,88	1,00
		268			0,48	0,28	0,26	0,40	0,12	0,00
CpG4	4	190	0.09	0.07	0,14	0,00	0,03	0,03	0,00	0,00
		205*			**0,02***	0,00	0,00	0,00	0,00	0,00
		207			0,84	1,00	0,97	0,97	1,00	0,94
		209*			0,00	0,00	0,00	0,00	0,00	**0,06***
CpE3	2	192	0.03	0.04	0,94	0,98	1,00	1,00	1,00	1,00
		194			0,06	0,02	0,00	0,00	0,00	0,00
CpE4	2	218	0.46	0.75	0,08	0,04	0,05	0,09	**0,88†**	**1,00†**
		230			**0,92†**	**0,96†**	**0,95†**	**0,91†**	0,12	0,00
CpG6	3	243	0.52	0.28	0,58	0,43	0,38	0,29	1,00	1,00
		245			0,12	0,02	0,18	0,11	0,00	0,00
		265			0,30	0,54	0,44	0,60	0,00	0,00
CpE8	1	111	0	0	1,00	1,00	1,00	1,00	1,00	1,00
CpG3	3	197	0.47	0.75	0,02	0,02	0,18	0,00	**0,88†**	**1,00†**
		211*			**0,06***	0,00	0,00	0,00	0,00	0,00
		216			**0,92†**	**0,98†**	**0,82†**	**1,00†**	0,12	0,00
I07-650	4	274	0.66	0.35	0,30	0,46	0,46	0,57	0,00	0,00
		280			0,50	0,48	0,36	0,34	0,12	0,00
		295			0,20	0,07	0,18	0,09	0,86	1,00
		297*			0,00	0,00	0,00	0,00	**0,02***	0,00

Supplementary table 3. Detected microsatellite haplotypes across six investigated populations.

Haplotype[1]	Switzerland		Croatia		North Macedonia	
	Contone	Orselina	Kast	Ozalj	Kalishte	Smolare
Cp1	1					
Cp10	1					
Cp14	2	1				
Cp15	7	2	7	6	5	
Cp17	1	2		2		
Cp18	2					
Cp20	1					
Cp21			1			
Cp22		1				
Cp30	2	2	1	1		
Cp31	1	1				
Cp33	4	8	14	11		
Cp34	2	3		2		
Cp4		1				
Cp40	1	1				
Cp41	2	1	2	2		
Cp44	1	1		2		
Cp45	1	2				
Cp48	1					
Cp49			1	1		
Cp5	5	7	2			
Cp50			1	1		
Cp54		1				
Cp55	1					
Cp57	1			1		
Cp59	1					
Cp6		1				
Cp60	1	1				
Cp61	1					
Cp62	1					
Cp63	1					
Cp65		1				
Cp66				1		
Cp67	1					
Cp68	1					
Cp69		1				
Cp70	1					

Cp71		1				
Cp73	1					
Cp74	1					
Cp75	1					
Cp77		1				
Cp78		1				
Cp8	1	4		1		
Cp87			1			
Cp9	1	1	1			
Cp90			1		37	29
Cp90mutB						2
Cp90mutA					1	
Cp95			3			
Cp104			1			
Cp205			1			
Cp206			1			
Cp209				1		
Cp210				1		
Cp211				1		
Cp213				1		
Cp215			1	1		
Total	50	46	39	36	43	31

[1]Previously described microsatellite haplotypes up to Cp104; Cp 205-215 are newly described in this paper.

Supplementary table 4. Vegetative compatibility type diversity and prevalence of *Cryphonectria hypovirus 1* (CHV1) in *Cryphonectria parasitica* populations sampled in different years in the study areas. For populations studied in previous years the original data sets were kindly provided by Dr. Daniel Rigling, Dr. Ljiljana Krstin and Dr. Kiril Sotirovski for populations from Switzerland and Italy, Croatia and Slovenia, and North Macedonia, respectively. All population diversity indices were recalculated from original datasets utilizing methodology used in this paper.

	Switzerland									
Collection date	1990-2010								2014	
Population, year	Lumino, 1990	Gnosca, 1990	Claro, 1996	Novaggio, 1996	Lattecaldo, 2003	Biasca, 2004	Gnosca, 2010	Pura, 2010	Contone	Orselina
N	86	62	50	40	47	49	97	97	50	52
No. of vc types	14	16	9	7	9	7	14	24	16	11
Richness rarefaction (n=40)	10.09	12.78	8.32	7	8.51	6.75	10.05	15.83	14.08	9.52
Most common vc type (%)	EU-5 (36.0)	EU-2 (44.0)	EU-2 (40.0)	EU-2 (40.0)	EU-2 (38.3)	EU-5 (36.7)	EU-2 (38.1)	EU-1 (24.7)	EU-2 (30.0)	EU-2 (38.5)
H' (95% C.I.)	1.94 (1.67-2.01)	2.18 (1.82-2.34)	1.64 (1.28-1.83)	1.48 (1.15-1.65)	1.70 (1.34-1.86)	1.56 (1.27-1.70)	1.91 (1.62-2.01)	2.57 (2.26-2.70)	2.22	1.70
H' rarefaction (n=40)	1.83	2.09	1.61	1.48	1.68	1.54	1.79	2.36	2.16	1.65
e (95% C.I.)	0.50 (0.44-0.63)	0.55 (0.48-0.69)	0.57 (0.48-0.73)	0.62 (0.55-0.80)	0.61 (0.53-0.77)	0.68 (0.58-0.80)	0.48 (0.42-0.61)	0.54 (0.45-0.64)	0.58 (0.51-0.73)	0.50 (0.44-0.64)
HV%	59	40	50	50	23	51	52	43	57.7	75

	Croatia & Slovenia					
Collection date	2006				2014	
Population, year	Ozalj, 2006	Samobor, 2006	Kal, 2006	Gornji Suhor, 2006	Kast	Ozalj
N	43	14	18	25	38	36
No. of vc types	5	6	7	6	8	14
Richness rarefaction (n=14)	2.78	6	5.14	5.14	6.15	6.91
Most common vc type (%)	EU-1 (83.7)	EU-2 (35.7)	EU-1 (50)	EU-1 (44)	EU-1 (47.1)	EU-2 (36.1)
H' (95% C.I.)	0.63 (0.29-0.90)	1.59 (1.09-1.71)	1.47 (0.96-1.69)	1.31 (0.71-1.58)	1.78 (1.40-1.96)	1.98 (1.404-2.20)
H' rarefaction (n=14)	0.52	1.59	1.34	1.24	1.55	1.63
e (95% C.I.)	0.38 (0.33-0.60)	0.82 (0.64-0.95)	0.62 (0.57-0.83)	0.62 (0.51-0.86)	0.66 (0.49-0.79)	0.52 (0.47-0.71)
HV%	44.1	42.8	72.2	44	38.5	33.3

	North Macedonia									
Collection date	1995-2010								2014	
Population, year	Frangovo, 1995	Trebenistha, 1997	Drazhevo, 1998	Bansko, 1998	Mokrievo, 1998	Smolare, 1998	Smolare, 2006	Drazhevo, 2010	Kalishte	Smolare
N	54	56	51	7	9	55	33	101	43	31
No. of vc types	1	1	1	1	1	1	1	1	1	1
Richness rarefaction	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Most common vc type (%)	EU-12 (100)	EU-12 (100)	EU-12 (100)	EU-12 (100)	EU-12 (100)	EU-12 (100)	EU--2 (100)	EU-12 (100)	EU-12 (100)	EU-12 (100)
H'	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
e	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
HV%	46	0	0	0	0	0	24.2	67	46.5	25.8

References for previous studies: Switzerland (Bryner & Rigling, 2012; Cortesi et al., 1998; Hoegger et al., 2000 and unpublished data for the populations Biasca and Lattecaldo); Croatia and Slovenia (Krstin et al., 2008, 2011); North Macedonia (Sotirovski et al., 2004, 2006, 2009; Bryner et al. 2013).