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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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

100 introduction of *C. parasitica* into Europe, are more diverse. In North Macedonia, Greece and
101 Bulgaria, for example, a single vc type (EU-12) was found to be dominant (Risteski et al.
102 2013; Sotirovski et al. 2004). On the other side, in over 60 years old established *C. parasitica*
103 populations in Croatia and Switzerland up to 24 different vc types were found (Bryner and
104 Rigling 2012; Ježić et al. 2018).

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

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

parasitica range in Europe (Table 1). For example, the first appearance of the disease and natural hypovirulence occurred in the three studied countries over a course of almost 50 years. In Switzerland *C. parasitica* was first recorded in 1948 and hypovirulent isolates were identified in 1975 (Heiniger and Rigling 1994). In Croatia these events occurred in 1955 and 1978, and in North Macedonia in 1975 and 1995, respectively (Heiniger and Rigling 1994; Robin and Heiniger 2001). Although this ~20 years of delay might be biased by the methodology of hypovirus detection, it is reasonable to assume that virulent isolates spread faster into new areas due to their more vigorous reproductive capacity. Since the Swiss populations are the oldest, and those in North Macedonia the youngest, we expected to detect a persistent negative gradient in *C. parasitica* and CHV1 diversity, as well as in CHV1 prevalence, from Switzerland to North Macedonia, a pattern which has been hinted at by previous studies (Bryner et al. 2012; Milgroom et al. 2008). These studies, however, used different markers and targeted either the fungus or the hypovirus. Here, we combined microsatellite and *vic* genotyping for the fungus and single-nucleotide polymorphisms (SNPs) for the virus. Using both classical markers important for the dissemination of CHV1 within *C. parasitica* populations (vegetative compatibility (vc) types), as well as microsatellite markers allowed the characterization of the six *C. parasitica* populations, two from each of the investigated countries. Beyond that, the inclusion of CHV1 analysis (including changes in prevalence in populations and SNP analysis) provided deeper insight into the spatial and temporal development of the *C. parasitica*-hypovirus pathosystem in Europe.

Materials and Methods

Cryphonectria parasitica sampling and isolation

Sixty to eighty randomly chosen chestnut blight cankers per location were sampled in 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 (Bisseger 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.

Vegetative compatibility type and microsatellite genotype determination

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

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

CHV1 sequencing

Complementary DNA (cDNA) was synthesized from RNA using either the GoScript™ Reverse Transcription System (Promega Corporation, Madison, USA) or the Maxima First Strand cDNA synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's protocols. PCR amplification of part of the ORF-A segment was performed with the primer pairs EP713-5 and R2280 (Allemann et al. 1999) or hvep-1 and EP721-4 (Bryner et al. 2012). The PCR products were sequenced by Sanger technology using the same primers as mentioned above. The obtained forward and reverse sequences were aligned and edited with the program CLC Main Workbench 7 (CLC bio, Aarhus, Denmark). 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 Kruskal-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).

Population differentiation

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

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

Temporal changes of vc type diversity

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

CHV1 prevalence and diversity

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

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

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

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

418 Taking all this in account, an invasion pattern of *C. parasitica* in Europe emerges. The
419 central European population, which is diverse and harbours many vc types and microsatellite
420 haplotypes, was established after the first introduction event in northern Italy in the late
421 1930s. Given the absence of significant geographical barriers, the central European
422 population subsequently expanded into neighbouring regions, such as southern Switzerland,

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

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

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

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

All our analyses of the viruses revealed that despite the fact that all strains are closely related, the North Macedonian CHV1 strains formed a distinct sub-cluster that was separated from the Swiss and Croatian strains. This seems to support the hypothesis of western European origin of south-eastern European CHV1 populations, with an observable bottleneck and founder effect, as suggested by much closer relationship between the North Macedonian CHV1 strains. This is in agreement with a previous study by Bryner et al. (2012) in which 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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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 $\rho = 0.541$, $p = 0.219$; Croatia & Slovenia: Spearman $\rho = 0.358$, $p = 0.460$; North Macedonia: Spearman $\rho = 0$, $p = 1$.

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 $\rho = 0.0176$, $p = 0.945$.

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 $\rho = 0.4501$, $p = 0.1064$.

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 evenness 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.

Populations	Switzerland		Croatia		North Macedonia	
	Contone	Orselina	Kast	Ozalj	Kalishte	Smolare
<i>C. parasitica</i> 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)
<i>1) Vc type diversity</i>						
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
<i>2) Microsatellite diversity</i>						
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 (0.8-0.9)	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.

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

²Number 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*).

³Frequencies 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 (*).

Population	Switzerland		Croatia		North Macedonia	
	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.

Population	Switzerland		Croatia		North Macedonia	
	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
<i>Pi</i>	0.009	0.009	0.007	0.013	0.008	0.004
<i>Hd</i>	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.

EU-type	Switzerland				Croatia			
	Contone		Orselina		Kašt		Ozalj	
	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 *.

Locus	<i>Na</i>	Allele	<i>H_T</i>	<i>G_{ST}</i>	Switzerland		Croatia		North Macedonia	
					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

¹Previously 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)
<i>H'</i> (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
<i>H'</i> rarefaction (n=40)	1.83	2.09	1.61	1.48	1.68	1.54	1.79	2.36	2.16	1.65
<i>e</i> (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
N	43		14	18		25
No. of vc types	5		6	7		6
Richness rarefaction (n=14)	2.78		6	5.14		5.14
Most common vc type (%)	EU-1 (83.7)		EU-2 (35.7)	EU-1 (50)		EU-1 (44)
<i>H'</i> (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)
<i>H'</i> rarefaction (n=14)	0.52		1.59	1.34		1.24
<i>e</i> (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)
HV%	44.1		42.8	72.2		44

	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).