Population Structure of *Cryphonectria parasitica* in Swiss Chestnut Stands

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ABSTRACT. In the southern part of Switzerland (Ticino), two chestnut *(Castanea saliva)* stands with natural populations of *Cryphonectria parasitica* have been investigated since August 1990. Development of the cankers, vegetative compatibility (v-c) group profiles and distribution patterns of hypovirulence were determined. A total of 850 sprouts with 2 cm dbh were examined regularly in two plots of 50 m x 50 m. Growth of the cankers was measured and strains of *C. parasitica* were isolated. The number of reported cankers increased from 411 (August 1990) to 619 (April 1992). Whereas 315 (37.1%) trees were infected in August 1990, 420 (49.4%) trees were diseased in April 1992.

Vegetative compatibility groups and reactions on Bavendamm's medium were determined for 148 isolates from randomly chosen cankers. Eighty isolates showed normal culture characteristics and a positive Bavendamm reaction, while 68 had reduced pigmentation and sporulation and were Bavendamm negative. One-hundred-nineteen of the isolates were assigned to three predominant v-c groups and showed a high percentage of hypovirulence (50%). The remaining 29 strains belonged to 19 v-c groups; only 28% of them were hypovirulent. Canker growth rate of the 148 isolated cankers was measured. Hypovirulent cankers showed a significantly slower mean growth rate than the cankers where virulent isolates were sampled.

Vegetative compatibilty groups and hypovirulent strains of C. *parasitica* appear to be randomly distributed in the two plots. Aggregation of certain v-c groups or of hypovirulent strains was found neither in single sprout clusters nor on single chestnut stems.

Since 1948, chestnut blight, caused by *Cryphonectria parasitica* (Murr.) Barr, spread widely in the European chestnut *(Castanea saliva* Mill.) stands of Ticino (southern Switzerland). Natural occurrence of hypovirulent strains of the pathogen led to a decline of the blight severity (7). To use hypovirulence for biological control, more knowledge is required about diversity and spatial distribution patterns of the vegetative compatibility (v-c) groups and of the hypovirulent strains of *C. parasitica* in the chestnut stands.

Random distribution of a high number of different v-c groups in a small area is a significant barrier for dispersal of hypovirulence. One-hundred-seventeen *C. parasitica* isolates from southern Switzerland, tested by Bazzigher et al. (5), belonged to one of five v-c groups; seven isolates were solitary. Some of the isolates formed anastomoses with several v-c groups.

This preliminary report describes the population structure of virulent and hypovirulent *C. parasitica* strains in two European chestnut stands in southern Switzerland. The objective of this study was to characterize the distribution pattern of *C. parasitica* and disease development.

MATERIALS AND METHODS

Study sites were located in the Ticino (southern Switzerland) at Gnosca and Lumino where the European chestnut *(C. saliva)* is the most common deciduous tree species. The two sites were clearcut in 1984 and showed abundant chestnut regeneration. Each plot was about 50 m x 50 m square and contained 400-450 living chestnut sprouts, growing from 36 (Gnosca) and 40 (Lumino) stumps, respectively. The diameter of the sprouts at breast height ranged from 2-15 cm at the beginning of the investigation. There were no other competing hardwood species present in the two plots.

In the summer of 1990, all the chestnut sprouts were numbered and the location of each sprout cluster was mapped. Standing dead trees were removed. From August 1990 to April 1992, the two plots were examined seven times; all sprouts were checked for blight incidence and tree survival. All cankers up to 2 m above ground level were noted and their length measured vertically. New cankers caused by *C. parasitica* were recorded as soon as they were observed. Dead trees and the cause of mortality were noted.

Mass isolates of *C. parasitica* mycelium were taken by removing bark samples from the margin of randomly chosen cankers in August 1990. C. *parasitica* was isolated as described by the method of Bazzigher et al. (5) and maintained on slants of Difco potato-dextrose agar supplemented with L-methionine (100 mg/L) and biotin (1 mg/L) (PDAmb) at 4 C. Cultural characteristics were used to distinguish between virulent and hypovirulent isolates. Mycelium was grown on PDAmb at 25 C under white fluorescent light (2500 lux) with a 14-hr photoperiod for 2 wk. Strains that produced white mycelium with no or only few pycnidia were considered hypovirulent, whereas the isolates that produced many pycnidia were considered virulent. Additionally, phenoloxidase activity was tested using Bavendamm's medium (12). After three days the Bavendamm medium showed no discoloration with hypovirulent strains but a brown discoloration with virulent strains.

Tests for v-c groups were performed as described by Anagnostakis et al. (2). Each isolate was initially paired with three virulent tester strains belonging to the most common Swiss v-c groups (I, II, III). The compatible reaction showed confluent mycelia at the interface of the two strains. If barrage lines were formed with all of the three testers, the isolate was tested with nine additional virulent v-c tester strains representing the Swiss v-c groups IV and V and the seven strains that could not be assigned to any v-c group by Bazzigher et al. (5). If the isolates did not show any compatible reaction with these testers, they were paired with each other.

All of the hypovirulent strains were tested for their conversion capacity, i.e. the ability to transmit the hypovirulence factors to a virulent isolate, represented by a change of the culture characteristics. Conversion tests were performed by placing a virulent and a hypovirulent strain 1 cm apart from each other in a petri dish (9-cm diameter) containing PDAmb. The dishes were incubated at 25 C under white fluorescent light (2500 lux) with a 14-hr photoperiod. If no conversion occurred a ridge of pycnidia was formed by the virulent strain along the contact zone of the two strains. Each virulent isolate was tested with five hypovirulent strains belonging to the five Swiss v-c groups (M770 = I, M936= II, M930= III, M922= IV, M972=V) (5). All the hypovirulent isolates were paired with five virulent tester strains representing the Swiss v-c groups (M779= I, M774.119 M919 = III, M933= IV, M959 = V) to test for conversion capacity. C. *parasitica* strains that could not be assigned to one of these groups were paired against each other.

The diversity of *C. parasitica* populations was calculated as follows:

$D = S/N$ S=number of v-c groups, N=number of isolates

The population structure was further characterized by Shannon's diversity index (13):

$$H' = -\sum_{i=1}^{s} p_i \log_e p_i$$

p_i is the portion of the whole sample represented by each phenotype $(n_1/N, n_2/N, \ldots, n_s/N)$ from a population with S phenotypes.

RESULTS

A total of 850 chestnut sprouts were examined in the two plots at Lumino and Gnosca. In August 1990, 315 (37.1%) trees were infected by *C. parasitica,* whereas in April 1992, 420 (49.4%) stems had bark cankers. During this period, chestnut blight killed 43 (5.1%) trees in the two plots. In addition, 64 (7.5%) trees died for other reasons (Table 1).

Table 1. Observations at the two investigation sites, Lumino and Gnosca, Ticino August 1990–April 1992.

	Lumino	Gnosca
Number of living trees in Aug 90	410	440
Average dbh [cm] of the trees in Aug 90	5.5	4.4
Number of trees attacked by *C. parasitica* in Aug 90	153 (37.3%)	162 (36.8%
Number of trees attacked by *C. parasitica* in Apr 92	203 (49.5%)	217 (49.3%
Mortality of trees caused by *C. parasitica*, Aug 90–Apr 92	24 (5.9%)	19 (4.3%)
Mortality of trees by other reasons Aug 90–Apr 92	48 (11.7%)	16 (3.6%)
Number of lesions in Aug 90	199	212
Number of lesions in Apr 92	284	335
Number of *C. parasitica* isolates	86	62
Percentage of hypovirulent isolates	57%	31%
S/N	0.14	0.26
Diversity index H'	1.63	1.77

The number of reported lesions rose from 411 to 619 during the observation period, i.e., 208 new infections were found. From August 1990 to April 1992, 318 (77.4%) of the lesions that were already present 2 yr ago expanded more than 10% in length. The remaining 93 (22.6%) cankers did not grow.

In August 1990, 148 *C. parasitica* isolates were sampled in the two plots. Eighty (54%) isolates were virulent as judged by culture characteristics, i.e. pycnidia were formed and the reaction on Bavendamm's medium was positive. Sixty-eight (46%) of the isolates showed characteristics typical for hypovirulent strains. Cankers from which isolates were taken were measured regularly to determine the growth rate. The 68 cankers from which hypovirulent strains were isolated grew significantly slower than the 80 cankers from which virulent isolates were sampled (Figure 1).

One-hundred-thirty-two (89%) of the 148 *C. parasitica* isolates obtained from the two plots were assigned to one of six v-c groups (Table 2). Groups Ito V correspond to the classification of Swiss *C.* parasitica strains (5), while the sixth group was newly designated. About 80% (119 isolates) of all isolates fell into v-c groups I to III, while 13 (9%) *C. parasitica* strains were classified in the groups IV to VI. Sixteen isolates (11%) gave no consistent reaction or resulted in incompatible reactions with all tester strains and each other. They appear in Table 2 as "others."

Forty-eight percent of the *C. parasitica* isolates in v-c groups Ito VI showed hypovirulent cultural characteristics (i.e. reduced sporulation and negative, Bavendamm reaction) whereas only 25% of the rest had a hypovirulent culture type. Conversion tests showed that most of the hypovirulent strains are only able to convert those strains

Table 2. Vegetative compatibility (v-c) groups of 148 *C. parasitica* isolates (v=virulent, hv=hypovirulent) from the two plots Lumino and Gnosca.

V-c group	Lumino		Gnosca		Total	
	v	hv	v	hv	v	hv
I	12	21	17	11	29	32
II	0	10	1	2	1	12
III	16	5(+7)*	13	2(+2)*	29	7(+9)*
IV	2	3	1	0	3	3
V	0	1	1	0	1	1
VI	3	0	2	0	5	0
Others**	4	2	8	2	12	4
Total	37	49	43	19	80	68

*Hypovirulent strains that gave no significant barrier or anastomosis reaction at the interface when paired with virulent tester strains. They converted virulent strains of the two v-c groups II and III and are listed (in brackets) under group III (because many more virulent strains of v-c group III were found in the two plots).
**"Others" include single-occurring 16 isolates that could not be assigned to a distinct v-c group.

that were assigned to their own v-c group. Three hypovirulent strains found in the Lumino plot showed broad conversion capacity, i.e. they were able to convert virulent strains of at least three different v-c groups. Each virulent isolate found in Lumino and Gnosca was paired with the hypovirulent tester strain that belonged to the same v-c group; all virulent isolates were converted.

The distribution of v-c types and hypovirulent isolates is given for the Lumino plot (Figure 2). V-c groups and hypovirulence seem to be randomly distributed at both sites. Data were used to calculate diversity indices S/N and H'. By assigning 132 isolates to six v-c groups and the remaining 16 isolates to 16 different additional v-c groups the H' for both plots combined (Lumino and Gnosca) is 1.78 and D=S/N is 0.14. The diversity indices that resulted for each plot are listed in Table 1.

Eleven trees with two or more cankers were examined. Multiple occurrence of v-c groups on the same tree was found only three times.

DISCUSSION

The results of this study show that blight incidence is high in both 7-yr-old chestnut sprout clearcuts in the Ticino. At the end of the investigation period over 50% of the chestnut trees were infected by *C. parasitica*, exhibiting at least one canker. The actual incidence of chestnut blight was even higher, as cankers situated higher than 2 m from bottom were not registered. Although chestnut blight killed 43 (5.1%) chestnut trees in the two plots, *C. parasitica* is obviously not the only reason for the high mortality rate (12.6%) during the investigation period (20 months). Many trees died for other reasons. Competition among chestnut sprouts is probably the most important cause of mortality in these sprout clusters.

From randomly chosen cankers, 148 *C. parasitica* strains were isolated. In Gnosca and Lumino, the percentage of isolates with hypovirulent cultural characteristics was 31% and 57%, respectively. Nevertheless, mortality due to *C. parasitica* was not significantly different in the two plots.

Cankers from which virulent strains were isolated, showed a significantly larger vertical growth rate between August 90 and April 92 than did hypovirulent strains (Figure 1). In contrast, many of the cankers from which hypovirulent strains were sampled had very slow or no vertical growth during that period. Infection tests with virulent and hypovirulent isolates also resulted in faster growing cankers for virulent isolates (data not shown). The classification of the cankers was based on the isolates that were taken in August 1990. If our understanding of the epidemiology of hypovirulence is correct, one would assume that some of the virulent cankers were converted to hypovirulent cankers in the meantime. The relatively

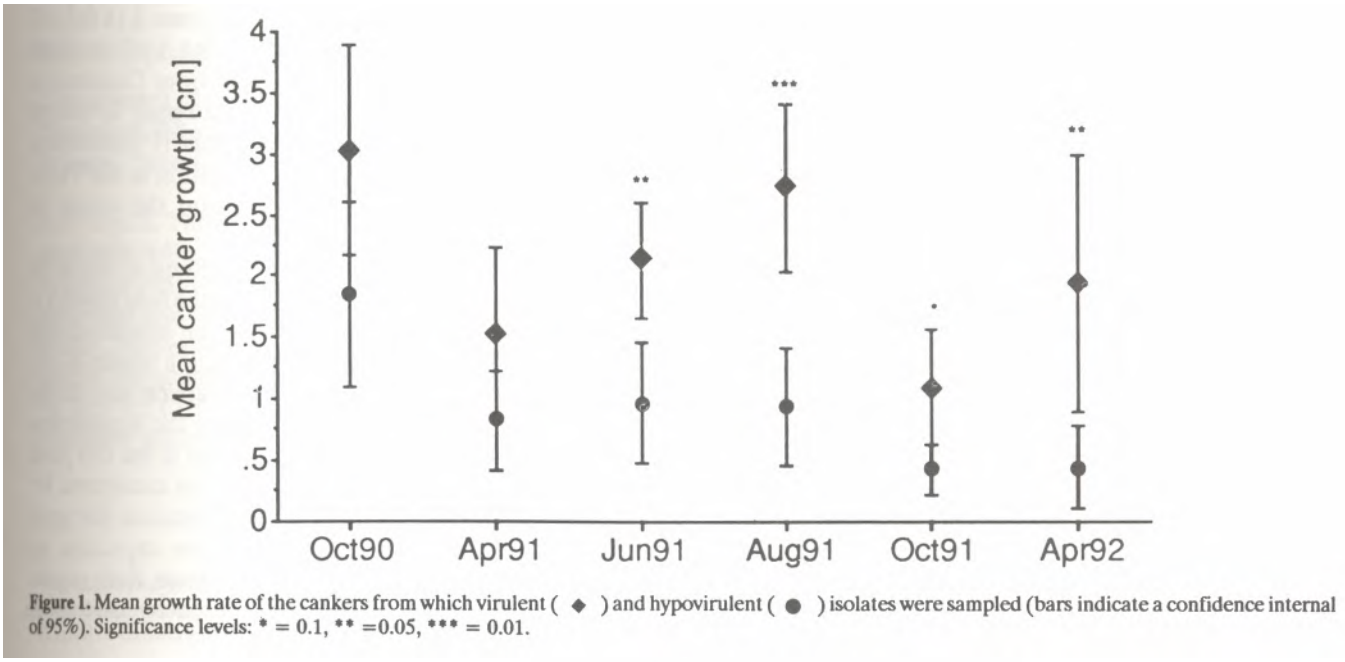

Figure 1. Mean growth rate of the cankers from which virulent (◆) and hypovirulent (●) isolates were sampled (bars indicate a confidence internal of 95%). Significance levels: * = 0.1, ** =0.05, *** = 0.01.

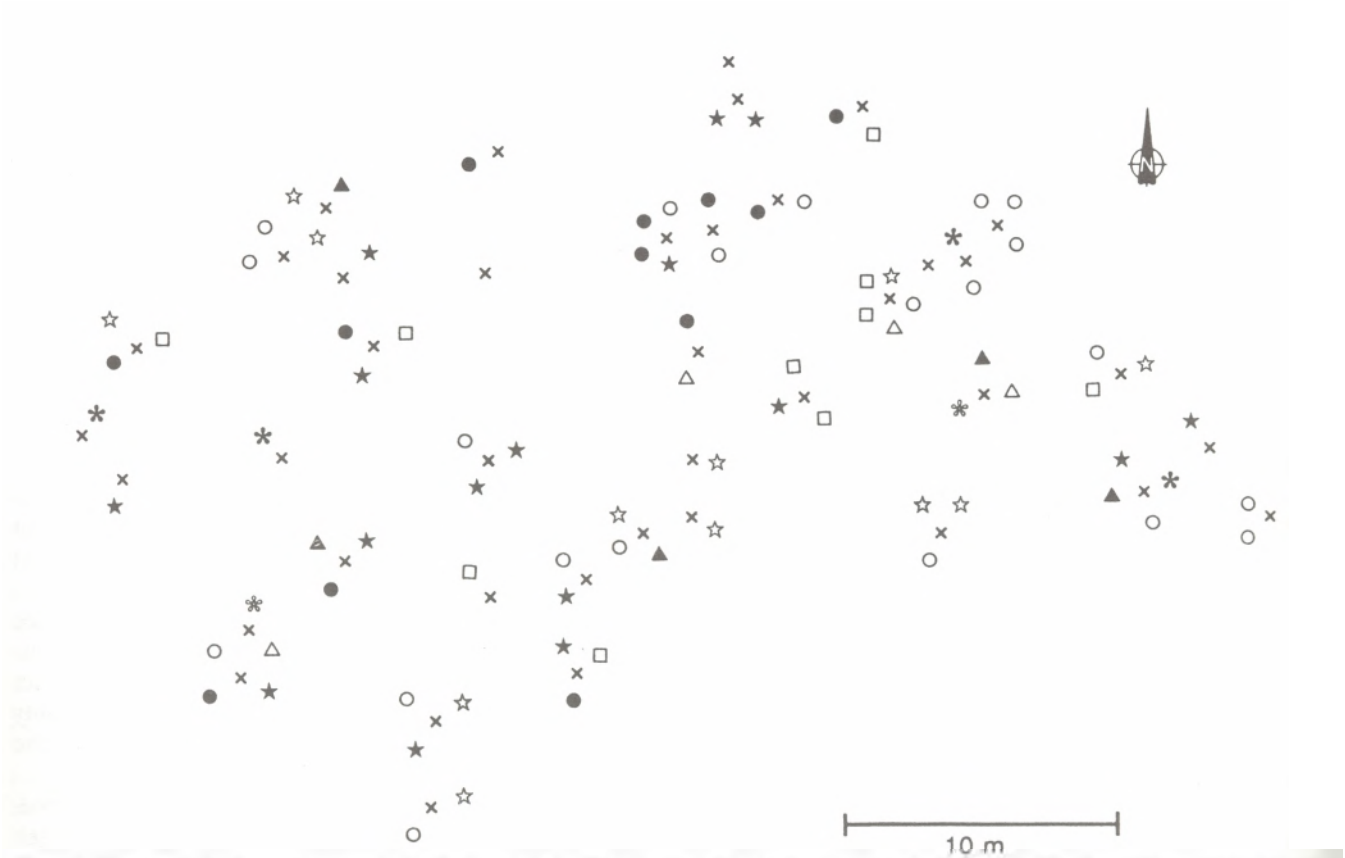

Figure 2. Mapping of the chestnut stumps (×) in the Lumino plot. Distribution of virulent and hypovirulent isolates of *Cryphonectria parasitica* belonging to different v-c groups (● = v-c group I, v; ○ = v-c group I, hv; ■ = v-c group II, v; □ = v-c group II, hv; ★ = v-c group III, v; ☆ = v-c group III, hv; ▲ = v-c groups IV–V, v; △ = v-c group IV–IV, hv; ✱ = v-c groups I–VI; ❋ = v-c groups I–VI, hv).

large standard error of the mean growth rate of the virulent cankers in April 1992 might be an indication that this has happened. All the cankers that were already sampled in August 1990 will be resampled in the fall of 1992 to see how many of them were indeed converted.

Most *C. parasitica* isolates could be classified in one of three v-c groups as defined by Bazzigher et al. (5), who found that v-c types I, II and III were the most common in the Ticino. Ten years later this finding is reconfirmed by this study. In addition to v-c groups IV and V, both of which were rare, a sixth group was found. It consists of five strains that are vegetatively compatible with each other and also with strain M788 of Bazzigher (5). One-hundred-thirty-two isolates fell into six v-c groups whereas 16 isolates could not be assigned to any v-c group. This amounts to an estimate of at least 22 v-c types for the two plots. In comparison with Bazzigher's data, which yielded 12 v-c types (5 groups and 7 single-occurring isolates) for the whole Ticino, our number is distinctly higher. There was some problem assigning hypovirulent strains to v-c groups. Usually the hypovirulent strains gave no consistent reaction (barrage or merging) at the contact zone with the neighboring strain (virulent or hypovirulent) or the reaction was very faint. Nine hypovirulent *C. parasitica* strains produced no barrage when paired with virulent tester strains for v-c groups II and III but were able to convert both of them. They were tentatively assigned to v-c group III.

In former studies, Grente (6) found 22 v-c types among 148 isolates sampled in France and Italy. Anagnostakis and Waggoner (4) reported 9 v-c groups among 49 Italian isolates, and 48 v-c groups among 272 cankers on 42 trees in Connecticut. Other investigations (1, 3,10) have shown that the diversity of v-c groups among *C. parasitica* isolates is much greater in North America than in Europe and responsible for limited spread of hypovirulence. Anagnostakis et al. (2) found a S/N between 0.14 and 0.41 and estimates of H' between 2.76 and 3.90 for North American *C. parasitica* populations from Connecticut, North Carolina, West Virginia and Virginia. In Europe the S/N was between 0.10 and 0.17 and the H' was between 1.47 and 2.74. In the present investigation in the Ticino both S/N = 0.14 and H'=1.78 confirm the values for Europe.

The spatial distribution of the v-c groups as well as the occurrence of hypovirulent cankers in the field appears to be random. This is consistent with the hypothesis that ascospores serve as the principal infection source (8, 10), assuming ascospores are dispersed relatively long distances. It also is interesting to note that the hypovirulent strains seem to be randomly distributed in the two plots. Hypovirulent strains cannot be spread by ascospores, but only by conidia or mycelium. It is hypothesized that hypovirulent strains with broad conversion capacities are responsible for dispersal of hypovirulence. Both vegetative compatibility and conversion capacity have to be

considered. Vegetative compatibility between two strains enables hyphal fusions and exchange of cytoplasmic factors. Conversion capacity is an indicator of the ability to build temporary or durable fusions between a virulent and a hypovirulent strain and to exchange hypovirulent factors (9). Conversion between a virulent and a hypovirulent strain occurs even between vegetatively incompatible strains, thus a hypovirulent strain can sometimes convert a virulent strain to a hypovirulent strain although the two strains are vegetatively incompatible.

All of the three hypovirulent strains that showed broad conversion capacity were found in the Lumino plot. This may be one reason for the higher portion of hypovirulent strains (57%) found in that plot compared with the Gnosca plot (31%). Hypovirulent fungal strains with broad conversion capacity are certainly important for the spread of hypovirulence in natural populations of *C. parasitica.* They may provide the appropriate inoculum for biological control on diseased trees or in infested stands. However, it is important to know more about the genetic background of the hypovirulent strains before using them as inoculum for biological control. If the introduced hypovirulent strains serve as male partners in sexual crosses and the resulting ascospores are of nonparental v-c types this can lead to an increase of v-c groups of the fungal population. Hypovirulent cankers are even able to produce pycnidia with virulent spores. Results of single conidiospore isolations showed that 8% (14) of the conidia produced on hypovirulent cankers are virulent.

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