Genetic Diversity of *Armillaria* spp. Infecting Highbush Blueberry in Northern Italy (Trentino Region)

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ABSTRACT

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Armillaria spp. are the causal agents of root rots of several woody plants, including highbush blueberry. Since 2003, highbush blueberry plants infected by *Armillaria* spp. have been found in Valsugana Valley, Trentino region, northern Italy. Our aim was to identify the *Armillaria* spp. involved in these infections, as well as possible sources of inoculum in blueberry fields. Samples of *Armillaria* spp. were collected from diseased blueberry plants in 13 infected blueberry fields, from bark spread along the blueberry rows, from infected trees in the vicinity of the fields, and from four forest locations. The identification of *Armillaria*

spp. was accomplished using a species-specific multiplex polymerase chain reaction method and by sequencing the rDNA at a specific locus. The differentiation between genotypes was performed by using simple-sequence repeat analysis. *Armillaria mellea* and *A. gallica* were the most frequently observed species infecting blueberry in the Valsugana Valley. Three to eight *Armillaria* genotypes were identified in each blueberry field. No individual genotypes were found in more than one blueberry field. Two-thirds of the genotypes found colonizing trees in the immediate vicinity of infected fields and two-thirds of the genotypes found colonizing the bark spread in blueberry rows were also isolated from blueberry plants in the field, indicating that bark used as mulch and infected trees surrounding the fields may be important sources of inoculum.

The Basidiomycetous genus *Armillaria* includes several species that live mostly as saprotrophs on wood and root debris in the soil (7). However, they also show variable degrees of pathogenicity and cause root rot on woody plants, including highbush blueberry (*Vaccinium corymbosum* L.) (4).

On blueberry, in the first stage of the disease, *Armillaria* spp. cause reduced plant vigor and infected plants develop small chlorotic leaves that redden prematurely in autumn. Later, the roots rot and the branches wilt. Infected plants usually die within a few months. White fan leaf mycelium is typically observed under the outer surface of the roots and rhizomorphs can be found inside and around the rotted roots. In fields and vineyards, the disease usually appears in patches of different dimensions, suggesting that the disease spreads through the soil, mainly via rhizomorphs originating from different inoculum sources (3,18,21). Genetic individuals, or genets, of *Armillaria* spp. are usually found in discrete patches that often include the root systems of several adjacent trees. Each dikaryotic (diploid) individual is thought to arise from a unique mating event and then grow vegetatively over a long period of time, across an ever-expanding area (23).

The Piedmont and Trentino regions are the main areas of highbush blueberry cultivation in Italy. In 2006, in the Trentino region of northern Italy, highbush blueberry was cultivated on an area of 69.65 ha, mostly (≈ 60 ha) in the Valsugana Valley, at altitudes of 400 to 1,000 m above sea level. In this region, blueberry bushes are planted in marginal agricultural areas close to the forest as well as in areas of intensive horticultural activity. The most common cultivar is Brigitta Blue (42 ha), followed by Elliot (6 ha). Blueberry plantations are small, with cultivated surface areas of

500 to 5,000 m². Plants are usually cultivated in rows and mulched with a deep layer (at least 10 cm) of coniferous bark. This bark is usually collected by the grower in the nearby forests. Bark is often kept for several months in heaps on the edges of the fields and new bark is added to the rows every 2 to 3 years, as the older bark decays.

In 2003, highbush blueberry plants showing typical symptoms of *Armillaria* root rot were first unequivocally identified in the Valsugana Valley. Fungal samples isolated from symptomatic plants were identified as *Armillaria gallica* Marxm. & Romagn. and shown to be the causal agent of the observed disease symptoms (20). Other than in Italy, *Armillaria* spp. have been reported to be pathogens of highbush blueberry in the United States. Specifically, *A. mellea* (Vahl) P. Kumm. and *A. ostoyae* (Romagn.) Herink have been isolated from infected plants in the United States (4).

Armillaria spp. infections in newly planted fields and vineyards often occur after forest trees or old plants with *Armillaria* root disease have been removed. In fact, infected roots that remain in the ground serve as a source of inoculum for infections of plants planted in these fields in later seasons (18). Coniferous bark is a good substrate for the development of *Armillaria* spp. (17). This implies that *Armillaria* spp.-infected bark used as mulch may represent another potential source of inoculum. The movement of soil, root and wood debris, infected plants, and tools and farm machinery from infected to uninfected areas may also help spread this disease. The infection in a vineyard or orchard can also originate from rhizomorphs that have grown from infected trees and stumps in the immediate vicinity.

No effective control agents are available for use against *Armillaria* spp. (1). Only agronomic practices, such as uprooting and removing all infected roots, long rotations, and fallowing rotations that allow the degradation of all infected root debris may help reduce the availability of *Armillaria* inoculum and the risk of new blueberry plants becoming infected (4).

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The aims of this research were to (i) identify the *Armillaria* spp. infecting highbush blueberry in the Valsugana Valley and (ii) determine the potential sources of inoculum in and around blueberry fields. To investigate those issues, a species-specific multiplex polymerase chain reaction (PCR) was developed and a genetic analysis based on microsatellite typing was conducted on samples of *Armillaria* spp. collected in the Valsugana Valley.

MATERIALS AND METHODS

Identification of infected highbush blueberry fields, monitoring of disease development, and collection of samples of *Armillaria* spp. The study was conducted in the Valsugana Valley, which is home to ≈350 blueberry fields. Two fields (05BV and 08BV) in which *Armillaria* root rot was first reported were monitored annually (in October) from 2003 through 2006, to assess the presence of symptomatic plants and plants that had been killed by *Armillaria* spp. in order to evaluate the development of the disease over time. Between June and September 2006, fields potentially infected by *Armillaria* spp. (plants displaying poor shoot growth, premature reddening, and dieback) were identified based on information provided by local growers and crop advisors. We inspected all of these potentially infected fields to verify the presence of signs of *Armillaria* root rot (mycelial fans and *Armillaria* rhizomorphs on roots). In October 2006, when aerial symptoms of root rot became more evident (premature reddening of leaves), the numbers and the percentages of stunted and dead plants in each of these fields were recorded. The presence of *Armillaria* spp. in stunted and dead plants was confirmed by removing the bark from the roots and root collars to expose any mycelial fans and rhizomorphs that might be present. For each of the examined fields, records were also collected concerning (i) the blueberry cultivar, the year of transplanting, and the year in which the first *Armillaria* symptoms were recorded by the grower; (ii) the possible inoculum sources inside and in the immediate vicinity of the cropped area (infected trees or stumps, bark heaps, or bark spread along the crop rows); (iii) the previous crop and the type of vegetation surrounding the blueberry crop; and (iv) whether the roots and stumps of the previous crop had been buried in the field (Table 1).

In October 2006, fungal samples (pieces of mycelial fans and rhizomorphs) were collected from infected plants in the selected

fields (Fig. 1), after removing the bark from the blueberry roots and root collars to expose the pathogen. At least two samples were collected from each infected patch (defined as a group of at least two infected plants near one another) in each field. If present, samples of *Armillaria* spp. were also collected from bark used as mulch in the blueberry rows and from the bark heaps that are sometimes found at the edge of blueberry fields. Other plant species growing close to the blueberry fields and infected by *Armillaria* spp. were sampled as well. The sites in the forests from which growers usually collect bark to spread on blueberry fields were visited and checked for the presence of mycelial fans and rhizomorphs of *Armillaria* spp. Samples were collected from four main forest locations (forest 21- and 22BV and 21- and 22AV). The samples were stored at -80°C until they could be analyzed.

DNA extraction. Mycelia or rhizomorphs of *Armillaria* spp. from the above samples were freeze dried and homogenized in an MM300 homogenizer (Retsch, Haan, Germany). DNA was extracted from 3 to 10 mg of mycelia or rhizomorphs, as described by Gobbin et al. (8) for their high-throughput method, using NucleoSpin multi-96 kits (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Instead of one final elution step of 180 µl, two elutions of 90 µl each were performed.

Multiplex PCR for the identification of *Armillaria* spp. A multiplex PCR reaction for differentiating between *A. mellea*, *A. gallica*, and *A. ostoyae* was set up. *Armillaria* 18S rRNA, internal transcribed spacer 1 (ITS1), 5.8S rRNA, ITS2, and 28S rRNA sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank with the accession codes AY213587 and AY509188 (*A. mellea*); AY213571, AY509172, and AY509173 (*A. gallica*); AY213554, AY509156, and AY509157 (*A. ostoyae*); AY781248 (*A. borealis* Marxm. & Korhonen); and AY213590 (*A. tabescens* (Scop.) Emel.) were aligned using ClustalW (28). The universal *Armillaria* primer pair AITSF/AITSR was designed based on conserved regions common to all five previously mentioned *Armillaria* spp. For the identification of *A. mellea*, the primer combination AMEL3/ITS4 was used (25). The primer pairs AgPR1F/AgPR1R and AoPR1F/AoPR1R were designed based on species-specific polymorphic regions of *A. gallica* and *A. ostoyae*, respectively (Table 2). Primer specificity was tested using 5 µl of DNA extracted from the first elutions of

TABLE 1. Fields with *Armillaria* spp.-infected blueberry plants in the Valsugana Valley (Trentino, Italy)^y

Code	Location	Cultivar	Planting year	Symptom year ^z	Possible inoculum sources	Previous crop	Surrounding fields
01AV	Field	Brigitta Blue	1997	5	Hazelnut, walnut stumps	Blackberry	Forest/fields
02AV	Field	Brigitta Blue/Elliott	2003	3	Chestnut trees/stumps, bark mulch, bark heap	Meadow, chestnut	Forest
01BV	Field	Brigitta Blue	1998	3	Bark mulch	Pear	Fields
02BV	Field	Brigitta Blue	1997	4	...	Pear, meadow/pasture	Fields
03BV	Field	Brigitta Blue	2003	3	Plum tree, bark mulch	Meadow/pasture	Fields
04BV	Field	Brigitta Blue	2000	4	Bark mulch, bark heap	Meadow/pasture	Fields
05BV	Field	Brigitta Blue	1999	3	Cherry trees/stumps, bark mulch	Meadow, vineyard	Fields/meadow
06BV	Field	Brigitta Blue	2001	4	Chestnut, hazelnut trees, bark mulch	Forest, chestnut (buried roots and stumps)	Forest
07BV	Field	Brigitta Blue	1997	5	Cherry trees	Potato, cherry and plum	Fields/meadow
08BV	Field	Brigitta Blue	2000	3	Bark mulch	Black locust, hazelnut (buried roots and stumps)	Forest/fields
09BV	Field	Brigitta Blue	2000	5	...	Strawberry, zucchini	Fields/meadow
10BV	Field	Elliott	2002	3	...	Black locust, hazelnut, meadow	Forest
11BV	Field	Brigitta Blue	2000	4	Bark mulch	Meadow/pasture	Forest
21AV	Forest	...	...	...	Infected barks and logs	Forest	Forest
22AV	Forest	...	...	...	Infected barks and logs	Forest	Forest
21BV	Forest	...	...	...	Infected barks and logs	Forest	Forest
22BV	Forest	...	...	...	Infected barks and logs	Forest	Forest

^y Samples of *Armillaria* spp. were collected in 2006 from blueberry plants in the fields, from possible inoculum sources inside and in the immediate vicinity of the cropped area (infected trees/stumps, bark mulch, or bark heaps), and from forest sites, where growers collect bark to spread on their blueberry fields. The blueberry cultivar, year of transplanting, appearance of the first *Armillaria* root rot-like symptoms (recorded by the grower), possible sources of inoculum, previous crop, and type of vegetation surrounding the blueberry crop are indicated.

^z Year after planting that first symptoms were noted.

A. mellea CBS 102.91, *A. mellea* 18A and 18B (18), *A. tabescens* CBS 129.26, *A. ostoyae* ATCC 66009, and *A. gallica* 14A and 27A (18). The PCR mixture (final volume of 20 µl) consisted of the following reagents: 1× Qiagen Multiplex PCR Master mix containing HotStarTaq DNA Polymerase (Qiagen, Milano, Italy) and a primer mix consisting of 0.2 µM AgPR1F/AgPR1R, 0.2 µM AoPR1F/AoPR1R, 0.1 µM AMEL3/ITS4, and 0.05 µM AITSF/AITSR (Table 2). The amplification program consisted of an initial step at 95°C for 15 min; followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 90 s, and extension at 72°C for 90 s; with a final extension at 72°C for 10 min. This procedure was carried out in a PTC 100 thermocycler (MJ Research, Waltham, MA). Once validated for the seven above-mentioned strains, the multiplex PCR method for species identification was used to examine all of the 318 *Armillaria* samples collected in this study.

Microsatellite typing of *A. mellea* and *A. gallica* isolates. All fungal samples classified as *A. gallica* and *A. mellea* according to the multiplex PCR were further genotyped at the loci AgSSR21, -29, -62, -65, and -74 using *A. gallica* microsatellites (14). In a preliminary test, we found that these five simple-sequence repeat (SSR) loci could be transferred from *A. gallica* to *A. mellea*. The microsatellite PCR procedures were performed in a reaction mixture (final volume of 10 µl) consisting of 0.1 mM dNTP, 1.5 mM MgCl₂, 0.2 µM each microsatellite primer (14), 0.7 U of GoTaq polymerase, and 2 µl of *Armillaria* DNA from the second elution (DNA not quantified) in 1× GoTaq reaction buffer (Promega, Milano, Italy). The forward primers AgSSR21, -29, -62, -65, and

-74 were 5' labeled with the fluorescent dyes HEX, 6FAM, NED, HEX, and 6FAM, respectively (Applied Biosystems, Foster City, CA). The PCR amplification was performed according to the following program: an initial step at 94°C for 2 min; followed by 37 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 40 s, and extension at 72°C for 30 s; with a final extension at 72°C for 7 min. Amplicons from different loci were pooled on the basis of their size and fluorophore dye; equal volumes of PCR products of loci AgSSR21, -29, and -62 were pooled in one tube and PCR products of loci 65 and 74 were pooled in a second tube. Each pooled sample (1 µl) was mixed with 10 µl of formamide and 0.2 µl of ROX-500 ladder and separated by electrophoresis in an ABI PRISM 3730 capillary sequencer (Applied Biosystems). Raw data were analyzed using Genescan 3.7 and microsatellite fragment sizes were scored using Genotyper 3.6 (Applied Bio-

TABLE 2. Polymerase chain reaction primers targeting *Armillaria* rDNA that were used in this study

Primer	Sequence (5'→3')	Amplicon size (bp)
AgPR1F	GATTCGAAAGGGTAGGCTAACAACA	354
AgPR1R	CCTCGAAACCTAAGTAAACGGTTGA	...
AoPR1F	AAGAGCGAGTTAATAAGTGATTTG	283
AoPR1R	CAACTTTCGCTATAGGCCAAATCAA	...
AITSF	CAGGTAGGACTACCCGCTGA	934–961
AITSR	GTTCGATTAGTCTTTCGCCC	...
AIGSF	AGACGACTTGAATGGGAACGGG	470–500
AIGSR	TGCCGTTCAAAWWTTGAAC	...

Fig. 1. Map of the Valsugana Valley. Symbols represent the highbush blueberry orchards in which *Armillaria* root rot was found in 2006 and the forest sites where growers collect bark to mulch the blueberry plants.

systems). *Armillaria* isolates showing different SSR allelic patterns were designated as different genotypes. Conversely, individuals sharing the same SSR pattern were considered as having the same genotype. Shannon's equitability index (E_H) was used to assess population heterogeneity (19).

Identification of other *Armillaria* spp. To identify those *Armillaria* samples that could not be classified as *A. mellea*, *A. gallica*, or *A. ostoyae* in the previous multiplex assay, an rDNA sequencing assay was developed. *Armillaria* 26S rRNA and IGS1 sequences deposited in the NCBI GenBank with the accession codes AF163617, AF451823, and DQ115587 (*A. mellea*); AY509173, AF451830, and DQ336613 (*A. gallica*); AY509157, AF451815, and DQ115575 (*A. ostoyae*); AY509191, AF451835, and DQ115588 (*A. tabescens*); AY509184, AF451807, and DQ115583 (*A. cepistipes* Velen.); and AF451801, DQ115576, and AF243056 (*A. borealis*) were aligned using ClustalW. The universal *Armillaria* primer pair AIGSF and AIGSR (Table 2) was designed based on conserved regions of the rDNA of the above-mentioned *Armillaria* strains, encompassing variable regions. Primer specificity was tested on *A. mellea* CBS 102.91, *A. tabescens* CBS 129.26, *A. ostoyae* ATCC strain 66009, and *A. gallica* 14A. The PCR procedure was performed in a final volume of 20 μ l, consisting of 1 $\times$ PCR buffer, 0.1 mM dNTP, 1.5 mM MgCl₂, 0.2 mM each of the primers (AIGSF and AIGSR), 0.7 U of GoTaq polymerase (Promega), and 2 μ l of *Armillaria* DNA. The amplification was performed according to the program used for the microsatellite typing. The PCR products were purified using MultiScreen PCR₉₆ Filter Plates (Millipore, Vimodrone, Italy) according to the manufacturer's instructions. Cycle sequencing was carried out using the AIGSF and AIGSR primers and the BigDye Terminator kit (v3.1; Applied Biosystems). The cycle sequencing products were purified using the PCR Cleanup Filter Plates kit (Millipore) and loaded into an ABI 3100 Prism Sequencer (Applied Biosystems) according to the manufacturer's instructions. Sequences were edited using Sequencher (version 4.8; GeneCodes, Madison, WI) and then compared with sequences in the NCBI GenBank database using the Mega Blast program (2).

RESULTS

Identification of infected blueberry fields and disease development. Of the $\approx$ 350 blueberry fields in Valsugana, 23 were considered to be potentially infected based on information received from crop advisors and growers. Further inspection confirmed infection in 13 of these fields. Most of the fields in which infection was confirmed are located around the town of Borgo Valsugana, in the lowest part of the Valsugana Valley (Fig.

TABLE 3. Total number and percentages of healthy, stunted, and dead plants in the infected blueberry crops in the Valsugana Valley, Italy, in 2006

Code	Blueberry plants			
	Total no.	Healthy (%)	Stunted (%)	Dead (%)
01 AV	1,833	96.4	3.0	0.6
02 AV	530	83.4	16.4	0.2
01 BV	141	90.7	7.8	1.5
02 BV	198	76.2	18.7	5.1
03 BV	545	98.5	0.8	0.7
04 BV	302	93.7	5.3	1.0
05 BV	410	78.5	19.0	2.5
06 BV	571	92.3	7.5	0.2
07 BV	300	85.0	15.0	0.0
08 BV	547	74.5	20.0	5.5
09 BV	491	87.4	12.2	0.4
10 BV	231	81.8	16.9	1.3
11 BV	381	97.1	2.9	0.0
Average	...	87.3	11.2	1.5
Total	6,480	...	...	...

1). The average percentage of stunted plants in the infected fields was 11%. The highest rate of infection (20%) was observed in field 08BV (Table 3). The incidence of dead plants in each field was generally very low (0 to 2.5%), with values $>5\%$ in only two fields. All of the infected fields were planted between 1997 and 2003 and the first *Armillaria* root rot symptoms were generally observed 3 to 4 years after planting (Table 1). The disease usually started from individual blueberry plants in several points inside and at the margins of the fields and developed in patches. In all, 3 to 75 *Armillaria* samples were collected in each of the 13 infected blueberry fields (291 samples) and 2 to 14 *Armillaria* samples were collected from each of the forest locations (27 samples) (Tables 4 and 5). A 10 to 20% increase in the incidence of stunted plants was observed between 2003 and 2006 in the plots that were surveyed annually (05BV and 08BV). During the 4-year survey, the incidence of dead plants in these plots increased from 1.5 to 2.5 and from 4.5 to 5.5% in fields 05BV and 08BV, respectively.

Multiplex PCR for identifying *Armillaria* spp. The multiplex PCR procedure allowed us to differentiate between *A. mellea*, *A. gallica*, and *A. ostoyae*; it provided clearly distinguishable species-specific amplicons ($\approx$ 700, 354, and 283 bp, respectively) (Table 2; Fig. 2). The universal primer pair AITSF and AITSR allowed the PCR amplification ($\approx$ 950 bp) of the ITS1 and ITS2 loci of all four *Armillaria* spp. tested (Fig. 2, only one representative of each species is shown).

Identification of *Armillaria* spp. in blueberry fields and forest sites in the Valsugana Valley. After the validation of the multiplex PCR assay, the same approach was used to sort the 318 *Armillaria* samples collected in the Valsugana Valley by species. The dominant *Armillaria* spp. were *A. gallica* (in 242 samples) and *A. mellea* (in 71 samples). We did not identify any representatives of *A. ostoyae* (Tables 4 and 5). *A. gallica* was identified on blueberry plants (in 170 samples); in the bark heap in field 04BV (in 4 samples); on bark heaps in the forest (in 25 samples); on chestnuts, hazelnuts, stone fruit trees, and forest tree stumps (in 24 samples); and on the bark spread in the blueberry rows (in 19 samples). *A. mellea* was found infecting blueberry plants (in 65 samples), cherry trees (in four samples), and a bark heap in the forest (in two samples) but was not found on the bark spread in the rows. Five *Armillaria* samples could not be identified using the multiplex PCR procedure. DNA from these samples was sequenced at the 26S rRNA and IGS1 loci and the resulting sequences were compared with others in the GenBank database. The highest levels of sequence similarity (99%) were found with the species *A. gemina* Bérubé & Dessur. (one sample), *A. cepistipes* (one sample), *A. cepistipes* or *A. sinapina* Bérubé & Dessur. (two samples), and *A. calvescens* Bérubé & Dessur. or *A. sinapina* (one sample). For the last three *Armillaria* isolates, only an ambiguous species determination was possible. All of these rare species were collected from blueberry plants. Up to three *Armillaria* spp. coexisted in the same field (01AV) but, generally, one or two species were identified. In each field, a single species, either *A. mellea* (e.g., in 02BV) or *A. gallica* (e.g., in 02AV) usually dominated the population (Table 4).

Microsatellite typing of the *A. mellea* and *A. gallica* isolates. Microsatellite analyses were carried out exclusively on individuals of the most frequently observed species, *A. mellea* and *A. gallica*. Two of the five examined loci were monomorphic (AgSSR29, allele size = 155 bp and AgSSR65, allele size = 159 bp) while the remaining three were polymorphic. Up to three alleles were identified for each of the loci AgSSR21 (allele size range: 114 to 140 bp; number of alleles: 20) and AgSSR74 (allele size range: 116 to 144 bp; number of alleles: 20). The locus AgSSR62 (allele size range: 124 to 155 bp; number of alleles: 20) showed two alleles per individual. At the three polymorphic loci, 24 alleles were found to be common to both *A. gallica* and *A. mellea* while 13 were *A. mellea* specific and 23 were *A. gallica* specific. The comparison of the allelic patterns among *Armillaria*

isolates allowed the identification of 57 genotypes among the 313 *A. mellea* and *A. gallica* isolates collected (Tables 4 and 5). In all, 42 genotypes were identified among the samples collected in the crop and 15 genotypes were identified among the forest-collected isolates. Three to eight *Armillaria* genotypes were identified in each blueberry field (on blueberry plants and other inoculum sources). The genotypic diversity (E_H) within fields (samples from blueberry plants only) was <0.3 in five cases and reached 1.0 as a maximum. E_H within other inoculum sources was 0.15 to 0.55. In the two forest locations at which more than eight samples were collected, the genotypic diversity was moderate to high ($0.5 < E_H < 0.8$). No individual genotype was ever observed in more than one blueberry field or present in both field and forest sites. In seven of eight Valsugana fields in which *Armillaria* individuals were isolated from both blueberry and possible inoculum sources, one or two genotypes out of three to eight were found to be present on both blueberry plants and in the bark heap, bark in rows, and other infected plant species (all of them were trees) (Table 4). Two-thirds (five of eight) of the genotypes collected from trees in the immediate vicinity of the fields also infected blueberry plants. Similarly, two-thirds of the genotypes collected from the bark spread in blueberry rows were also identified on blueberry plants (Table 4). In one case (04BV), a genotype identified in the bark heap was also identified on blueberry plants and on the bark spread in the rows. A single genotype identified in field 02AV was found to be present on an old chestnut stump, on bark in the rows, and on blueberry plants.

DISCUSSION

In this study, a fast and reliable PCR-based approach for *Armillaria* spp. identification was successfully implemented. The multiplex PCR procedure allowed the identification of the three *Armillaria* spp. that had been previously reported on highbush blueberry (i.e., *A. mellea*, *A. gallica*, and *A. ostoyae*) (4,20). This species identification was achieved using a single reaction per root sample, representing an improvement over the previously established and time-consuming protocols based on singleplex

PCR procedures (25) and restriction fragment length polymorphisms (10). In 98% of the samples analyzed, either *A. mellea* or *A. gallica* was identified, greatly reducing the need for further identification procedures (IGS1 sequencing). In other studies, in which higher species diversity is expected, it would be preferable to test the multiplex PCR procedure on a larger set of known *Armillaria* spp. than those shown in Figure 2. As reported by Sicoli et al. (25), because of the great similarity in the sequences of *A. borealis* and *A. ostoyae* and between the sequences of *A. cepistipes* and *A. gallica*, we found the identification of these species to be problematic. The multiplex PCR procedure provided an internal positive control for each PCR reaction (*Armillaria* ITS, locus AITS). A totally failed multiplex PCR reaction is a warning sign indicating that the DNA extraction procedure may not have been successful or that polymerase inhibitors may have been present in the DNA eluate and does not imply the absence of *Armillaria* spp. on root samples (9).

Two of the five isolates that were not classified as *A. gallica*, *A. mellea*, or *A. ostoyae* were identified as *A. gemina* and *A. cepistipes* using IGS1 sequencing. The remaining three isolates were only ambiguously identified; they are most likely *A. cepistipes* or *A. sinapina* (two isolates) and *A. calvescens* or *A. sinapina* (one

TABLE 5. *Armillaria* spp., samples, genotypes, and genotypic diversity (Shannon index) identified in four forest locations in the Valsugana Valley, Italy, in 2006

Location code	Species ^y	No. of samples	No. of genotypes ^z	Shannon index
21AV	<i>A. gallica</i>	14	8	0.77
22AV	<i>A. gallica</i>	2	1	0.00
21BV	<i>A. gallica</i>	7	3	0.55
	<i>A. mellea</i>	2	2	1.00
22BV	<i>A. gallica</i>	2	1	0.00
Total	...	27	15	...

^y Multiplex polymerase chain reaction was used to identify *Armillaria gallica* and *A. mellea*.

^z Microsatellite markers were used to differentiate between the different genotypes of *A. gallica* and *A. mellea*.

TABLE 4. Location code, number of *Armillaria* spp. samples, species, number of genotypes, and genotypic diversity (Shannon index) in 13 blueberry fields on blueberry plants and on other sources surrounding fields (trees, bark in the rows, and bark heaps) in the Valsugana Valley, Italy, in 2006

Code	No. ^w	<i>Armillaria</i> spp. ^x	Blueberry plants			Other sources ^y				
			No. of samples	No. of genotypes ^z	Shannon index	No. of samples	No. of genotypes	Genotypes on trees	Genotypes on bark in rows	Genotypes on bark heap
01AV	18	<i>A. gallica</i>	11	6	0.70	4	2	a*, b	...	...
		<i>A. gemina</i>	1	...	...	...	...	...	...	...
		<i>A. cepistipes</i> or <i>A. sinapina</i>	2	...	...	...	...	...	...	...
02AV	52	<i>A. gallica</i>	32	3	0.22	19	2	c*, d	c*	...
		<i>A. calvescens</i> or <i>A. sinapina</i>	1	...	...	...	...	...	...	...
01BV	3	<i>A. mellea</i>	3	3	1.00	...	...	...	...	...
02BV	38	<i>A. gallica</i>	1	1	...	...	...	...	...	...
		<i>A. mellea</i>	37	3	0.09	...	...	...	...	...
03BV	16	<i>A. gallica</i>	9	1	0.00	7	3	e	f*, g	...
04BV	21	<i>A. gallica</i>	10	2	0.27	10	2	...	h*, i*	i*
		<i>A. cepistipes</i>	1	...	...	...	...	...	...	...
05BV	13	<i>A. mellea</i>	13	6	0.59	...	...	...	...	...
06BV	29	<i>A. gallica</i>	24	2	0.14	5	2	j*	k	...
07BV	10	<i>A. mellea</i>	6	3	0.61	4	2	l*, m*	...	...
08BV	75	<i>A. gallica</i>	72	2	0.10	1	1	...	n*	...
		<i>A. mellea</i>	2	1	0.00	...	...	...	...	...
09BV	4	<i>A. mellea</i>	4	3	0.75	...	...	...	...	...
10BV	9	<i>A. gallica</i>	9	4	0.46	...	...	...	...	...
11BV	3	<i>A. gallica</i>	2	2	1.00	1	1	...	o	...
Total	291	...	240	42	...	51	15	8 (5)	8 (5)	1 (1)

^w Number of samples.

^x Multiplex polymerase chain reaction was used to identify the *Armillaria* spp. *A. gallica* and *A. mellea*. The species *A. gemina* and *A. cepistipes*, as well as the ambiguously identified *A. cepistipes* or *A. sinapina* and *A. calvescens* or *A. sinapina*, were identified following 26S rRNA and IGS1 sequencing and the comparison of the resulting sequences with those in the National Center for Biotechnology Information GenBank database.

^y Different genotypes on other sources are marked with letters; asterisk (*) indicates that the same genotype was found also on blueberry plants.

^z Microsatellite markers were used to differentiate between the different genotypes of *A. gallica* and *A. mellea*.

isolate). *A. gemina*, *A. sinapina*, and *A. calvescens* are not yet reported in Europe (16), whereas *A. cepistipes* is both a European and an extra-European species (12). Therefore, if we theoretically exclude species that are not yet present in Europe, it is reasonable to assume that three of these five isolates are most likely *A. cepistipes*. If further studies will confirm the identity of *A. gemina*, we can hypothesize that it could have been introduced from North America with nursery plant material, which is a common practice in the studied area. As Kim et al. (12) also found, it is not possible to distinguish between those species using IGS1 sequences, and sequencing the ITS and 5.8S loci does not provide a more accurate identification of the species. The use of other DNA markers, such as amplified fragment length polymorphisms, may allow for more reliable identification in difficult cases.

As described above, the main species found in the 13 fields and four forest locations were *A. mellea* and *A. gallica*. The former is a species that is known to have a wide host range, which includes orchard trees (6,24), while the latter is mainly a forest species that infects stressed hardwood and coniferous trees (14). The present study confirmed this previous observation, because *A. mellea* was found on blueberry plants, on cherry trees (07 BV), and in two forest samples. The fields hosting a widespread *A. mellea* clone were surrounded by other orchards or had been previously cultivated, usually with fruit trees. As expected, *A. gallica* was identified among the forest samples but it was also identified as the dominant species in eight blueberry fields. These particular crops directly neighbor forests, suggesting that *A. gallica* may have migrated from the forest into these fields or may have been present in the fields before the blueberry plantation was established. *A. gallica* may also have been introduced into the field via infected coniferous bark used as mulch, because this species was found in this bark.

La Porta et al. (13) in 2006 identified several *Armillaria* spp. among the rhizomorphs and fruiting bodies they collected from 40 forest sites in Trentino. In their study, the most frequently isolated species was *A. cepistipes* (61%), followed by *A. ostoyae* (26%), *A. gallica* (7.5%), *A. mellea* (4.8%), and *A. borealis* (0.5%). The different results obtained in this study suggest that in each forest (and cultivated) area of the region there is probably a different predominant species of *Armillaria* (i.e., *A. gallica* and *A. mellea* in Valsugana Valley, which represents only a limited part of Trentino).

Microsatellite analysis allowed the study of the structures of the *A. mellea* and *A. gallica* populations. Up to now, *Armillaria* population structures were generally assessed using somatic incompatibility testing (26) and IGS sequencing (18). To the best of our knowledge, SSR markers have only been used once before for confirming the genetic homogeneity of *A. ostoyae* genets (30). Prior to this study, this method had never been used to examine *A.*

gallica. Five of the six *A. gallica* SSRs (14) were used successfully in this study, both for *A. mellea* and *A. gallica*. However, only three of these SSRs were polymorphic in the Valsugana Valley populations examined. This may be due to the relatively restricted geographical area in which the sampling was performed. The analysis of further Italian, other European countries, and global populations may reveal whether there is more diversity at those loci. The SSR markers AgSSR21 and -74 generated a complex banding pattern (more than two alleles per locus and concurrent one-base and two-base stuttering) that required a lot of attention during scoring. Despite their complexity, the determination of genotypes was still possible after cross-comparisons among banding patterns of different individuals. For studies of population genetics, the use of such SSRs is not recommended because a strictly diploid data set is often required.

The finding of more than two alleles per locus suggests polyploidization, locus duplications, or the sampling of more than a single genotype. This phenomenon has been previously observed in populations of *Plasmopara viticola* (22) and *Phytophthora infestans* (5,29), in which it was assumed to be due to tetraploidy. Based on the currently available information, it is not possible to verify any similar hypothesis for the *Armillaria* spp. in the Valsugana samples.

Stunted and dead blueberry plants were located in close vicinity of one another, possibly indicating that *Armillaria* spp. infections started from a single plant and then spread to the surrounding plants by means of rhizomorphs. This same pattern was repeatedly observed in each of the infected fields, suggesting that the disease spread from different inoculum points.

In each of the 13 fields, a plot-specific *Armillaria* genotype was sampled multiple times (up to 62 times in 08BV), indicating its widespread colonization of blueberry plants. The genotypes sampled less frequently were concentrated on single plants or groups of adjacent plants. That spatial pattern is consistent with the spatial structure reported by Worrall et al. (30) for *A. ostoyae* in an area in which a couple of more spatially restricted genotypes coexisted with one widely spread genotype. Pertot et al. (18), who analyzed the spatial distribution of *A. mellea* in the large Italian viticultural area of the Trentino region, also observed both a high level of genetic diversity and vineyard-specific genotypes. Individual *A. mellea* clones (defined as individuals that have the same IGS1 sequence and belong to the same SI group) were never found in different vineyards. Also, in the present study, individual *Armillaria* genotypes were never found in more than one field, even when fields were located only 30 m apart from one another (e.g., 09 and 10BV). This may reflect the slow growth rate of *Armillaria* spp., which can be only a few centimeters per year (11,21). Interestingly, the same genotypes were not even found in different fields belonging to the same grower (e.g., 01-02 BV and 09-10 BV), indicating that the movement of *Armillaria* spp. with infected plants and contaminated farm machinery and tools may be a rare event.

The spatial spread of *A. gallica* was found to be similar to that of *A. mellea* (data not shown), even though the two species have different growth rates and different pathogenicity patterns. *A. gallica* commonly produces a more extensive network of rhizomorphs in soil than *A. mellea*; therefore, it may have an advantage in terms of contacting new substrate and colonizing larger areas (15,27). On the other hand, *A. gallica* is considered to be a weak pathogen, whereas *A. mellea* is a virulent species on forest and orchard trees (6,14,15). These complementary characteristics probably give the two *Armillaria* spp. comparable spreading abilities in blueberry fields.

Armillaria spp.-infected trees growing in the proximity of recently established blueberry fields were assumed to be a potential inoculum source. This hypothesis was confirmed in four of the five fields in which infected trees were identified. One to two *Armillaria* genotypes were found on both infected trees and

Fig. 2. Multiplex polymerase chain reaction for distinguishing *Armillaria* spp. The universal primers AITSF and AITSR were used to amplify the rDNA of *Armillaria tabescens* (T), *A. mellea* (M), *A. gallica* (G), and *A. ostoyae* (O). Species discrimination among *A. mellea*, *A. gallica*, and *A. ostoyae* was made possible by species-specific amplicon sizes (Table 2); w, water control; l, 100-bp ladder.

blueberry plants. The presence of infected trees, preexisting the establishment of the blueberry fields, let us speculate a directional vegetative expansion of *Armillaria* spp. from these trees to the blueberry plants rather than the opposite direction. The presence of the same genotypes on trees and blueberry indicates that infected trees are a potential source of inoculum that must be taken into consideration when new blueberry plantations are established. Surrounding trees should be inspected and infected roots should be removed before new blueberry plantations are established. Agronomical practices carried out in the field in previous seasons should also be considered when selecting a blueberry plantation site. Stone fruit, apple and pear trees, and grapevines are particularly susceptible to the disease and should be carefully inspected. Burying infected roots and stumps of old fruit or forest trees before the new planting could represent a dangerous practice, which may promote the spread and development of *Armillaria* populations. For example, roots of hazelnut trees and black locust trees had been previously buried in field 08BV, which was the most seriously infected field, and this practice probably promoted the spread of the pathogen in the soil (one genotype was widespread throughout the plot) and the subsequent infection of blueberry roots.

Similar to old infected trees, bark heaps at the edges of the field (in one of the survey locations) and bark spread in rows (in four of six cases) were shown to be additional sources of inoculum. In these cases, one or two of the genotypes identified on the bark heap or on the bark in the rows were also identified on blueberry roots. *A. gallica*, which produces abundant rhizomorphs, seems to grow better than *A. mellea* on coniferous bark. It produces nets of rhizomorphs that hold bark pieces together. *A. gallica* was identified in 48 bark samples (bark heaps in the field, in the forest, and on bark in the rows). In contrast, two samples of *A. mellea* were isolated from bark heaps in the forest, and this species was never identified on bark in the rows or bark heaps in the field. Visual bark inspection, vapor sterilizing the bark, or inoculating the bark with biocontrol agents before spreading it along the crop rows may help reduce the spread of *A. gallica* into blueberry fields.

According to our sampling, no individual genotypes were recovered from both bark in the fields and bark in the forest. It should be mentioned that the bark that was spread in the rows was collected in the forest before we made our bark collections, thus reducing the probability of our finding genotypes common to both areas. Some genotypes observed on blueberry were not found on any of the other possible inoculum sources in or around the crop. This may be due to the relatively small number of samples collected from the bark heaps, the bark in the rows, and the surrounding trees. Nevertheless, it is also possible that the genotypes that were recovered from blueberry but not from the putative sources of inoculum may be indigenous to the area, having colonized the area prior to the establishment of the blueberry fields.

This research demonstrates that highbush blueberry is susceptible to *Armillaria* spp. infection but, to date, in the Trentino region of Italy, this infection has been limited to a few fields. This study also included the development of a rapid tool for the identification of *Armillaria* spp. that can easily be implemented in studies involving other infected areas and other hosts. Microsatellite typing showed that the *A. mellea* and *A. gallica* populations in the Valsugana Valley are genetically diverse and that individual genotypes are spread over relatively small areas.

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