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DOTTORATO DI RICERCA IN SCIENZE DELLA TERRA E DELL'AMBIENTE

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Incidence and effects of *Colletotrichum acutatum* J.H. Simmonds 1968, in *Dryocosmus kuriphilus* Yatsumatsu (*Hymenoptera Cynipidae*) galls on *Castanea sativa* Mill.

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Summary

Abstract

Castanea sativa Mill. (genus *Castanea*, family *Fagaceae*), is a typical nut crop of the Mediterranean basin and one of the most common forest trees in the hilly parts of Italy (Borghetti *et al.,* 1986). Therefore, chestnut tree is economically and environmentally important in Europe and in Italy. It is distributed from Southern Europe and North Africa, to North-Western Europe and eastward to Western Asia, with an altitudinal range between 200 and 1800 m, depending on the latitude and site aspect (Conedera *et al.,* 2016).

Italy is the largest exporter of chestnuts for the quality of trade, while it is second for quantity traded; the export price of Italian chestnut is, in fact, higher than that of Asian chestnuts. The ISTAT data show that chestnut production is mainly concentrated in Campania, Calabria, Tuscany, Lazio, Piedmont, Emilia-Romagna and Lombardy.

Regarding chestnut for wood production, in Italy about 10.5 out of 30 total million ha are occupied by forests; overall, over 75% of Italian chestnut is intended for timber production.

In Lombardy, *C.sativa* is present in Como, Lecco, Sondrio, Varese, Pavia and Brescia provincies mainly in mixed forests (Boriani *et al.,*2013). *C.sativa* is widespread both along the alpine and prealpine chains and along the Apennines.

Sweet chestnut is one of the best example of a forest tree species threatened by invasive pathogens and insect pests, with major impacts caused by *Cryphonectria parasitica* (Murrill M.E.) Barr, *Phytophthora* spp. and *Dryocosmus kuriphilus* Yasumatsu (Asian Chestnut Gall Wasp) (Kato and Hijii, 1997; Reale *et al*., 2014; Turchetti *et al*., 2008).

Many are pathogenic fungi that can cause lesions in the chestnut plant, and the occupation of potential micro-habitats of pathogens by avirulent endophytic might prevent invasion or infection by disease causing fungi (Carroll, 1988; Minter, 1981).

Chestnut fruit production and quality have strongly declined during the last years by the influence of the cumulative effects of either the spread of the Asian chestnut gall wasp or the irregular weather (Battisti *et al*., 2014). The impact from *D. kuriphilus* in Europe on yield reduction in *C.sativa* was estimated as moderate in the last available review (EFSA Panel on Plant Health, 2010)

moreover, these data are confirmed by the study of Battisti *et al*., 2014 where it is observed evidence of a relationship between gall density and yield loss.

The chestnut gall wasp *D.kuriphilus*, one of the most important pests of chestnut worldwide, causes significant damage to European chestnut, as well as to the susceptible Euro-Japanese hybrids (EPPO, 2005). Native to China, it is the only member of the Cynipini tribe which attacks the genus *Castanea*. Since 2006, have been carried out biological controls of the pest, releasing a natural antagonist, the insect *Torymus sinensis* Kamijo.

Chestnut gall wasp causes on *C.sativa*, galls on young twigs, on leaf petioles or on leaves. They measure 5-20 mm in diameter and are green or rose-colour and are readily detected on plants or part of plants, while eggs and first instars larvae inside the buds cannot be detected by simple visual inspections. The creation of galls results in preventing or inhibiting the development of normal shoots and the production of abnormal plant structures (Maltoni *et al*., 2012).

In cases of severe *D.kuriphilus* attacks, mortality of young plant or weak plants occurs, strongly reducing the quality and quantity of timber (Kato and Hijii, 1997; Maltoni *et al*., 2012), nuts and flowers.

In 2014, during the insect monitoring, the occurrence of *Colletotrichum acutatum* J. H. Simmonds in necrotized galls of *D. kuriphilus* in chestnut stands was recorded for the first time in Italy (Gaffuri *et al.*, 2015). Thus, the overall goal of this work, has been to investigate the necrotized galls in order to isolate and identify the causative agent and its potential action against the parasite insect.

Consequently, it has been necessary to organize a monitoring and sampling of shoots bark and galls on *C.sativa*. Subsequently, isolation and identification of pathogen and its characterization using both morphological and molecular techniques was carried out. Moreover, it was necessary a molecular and bioinformatics approach, based on data available in GeneBank.

For the first time, we discoved *Colletotrichum acutatum* in Lombardia and Trentino orchards, and from monitoring data, this fungus was found to be spread in all chestnut-growing areas monitored on necrotic galls. Furthermore, during this survey, single or few nuts showed a still undescribed symptom: a clear and sometimes intense pink coloration of the endosperm. This was generally the main cause of nut decay although sometimes associated with brown rot symptoms (Maresi *et al*.,

2013). “Pink rot” has never been reported previously as a chestnut rot symptom; therefore, an investigation was carried out to clarify the causal agent of this new symptom.

C.acutatum is commonly found on cultivated and weeds plants and it was associated to a typical symptomatology, anthracnosis, with subsequent and progressive necrosis of green tissues. However, these symptoms were not observed on chestnut.

Since this study represents the first reporting of *C.acutatum* in chestnut and galls of *D.Kuriphilus*, other molecular analysis on barcode genes in addition to ITS were necessary to confirm the identification of *Colletotrichum*. Moreover, the molecular analysis showed the presence of the holotype *Glomerella acutata*, an interspecific hybrid between *C.acutatum* and *C.fioriniae* (Vaillancourt *et al*., 1992).

Colletotrichum spp. have a wide host range and anthracnose symptoms appear on young and old trees on leaves, fruits and flowers of several crops of agronomic interest and on several herbaceous plants (Damm *et al*., 2012).

Colletotrichum species, have also been recorded endophytically (Dingley and Gilmour 1972, Wang *et al*., 2008; MacKenzie *et al*., 2009) and some isolates (*Colletotrichum acutatum* var *fioriniae*) have been detected in mealybugs (Marcelino *et al*., 2009). Isolates of *C.acutatum* var *fioriniae* were also recovered from *Tsuga canadensis* (L.), a common species in forest in the northeastern United States (Marcelino *et al*., 2009).

The genus *Colletotrichum* has undergone frequent taxonomic changes in the past decades with the merging and addition of many species (Baroncelli *et al*., 2017) and now are recognized as species *complex* (Damm *et al*., 2012). Members of the *C.acutatum* species complex cause both pre-harvest and post-harvest diseases (Shi *et al*., 1996).

A total of 360 samples from shoots and necrotic or healthy galls were taken in order to determine the presence of fungi. Shoots (as leaves) showed no symptoms but in some cases, the collected galls were necrotic. From the isolation it was possible to identify the presence of cultures referred to *Colletotrichum* sp. in nineteen area out of the monitored forty. Positive isolation of other fungi as *Trichoderma* sp, *Fusarium* sp., *Cryphonectria parasitica* and *Gnomognopsis* sp. were generally obtained in all the areas. *Colletotrichum* coltures were obtained from the province of Trento (Serci, Drena, Pranzo, Castione); Bergamo (Le Piane and Valmoresca); Como (Torno, Gravedona, Albese and Livio) and Lecco (Monte Barro, Oggiono, Ballabio, Introbio, Primaluna and Barzio).

By molecular characterization *Colletotrichum* isolates associated with galls and shoots bark of *C. sativa* have been identified as *C. fioiriniae* and the analysis of rRNA gene-ITS, TUB and CAL nucleotide sequence data strongly supported these results.

Molecular methods are used successfully in differentiation between the species of *Colletrotrichum acutatum* species complex and analysis of the nucleotide sequence of the internal transcribed spacing (ITS) of the ribosomal DNA (rDNA) from genes of tubulin (TUB), and calmodulin (CAL) show the genetic complexity of *Colletotrichum* isolates. Results showed that a phylogenetic analysis based on a single gene, could not distinguish the species specificity in *Colletotrichum* complex (Mahdi *at al.,* 2015); only the multigene phylogenetic analysis allow it (Cannon *et al*., 2012; Damm *et al*., 2010, 2012, 2014; Weir *et al*., 2012).

C. *fioriniae*, as shown in Baroncelli *et al*., 2014, belongs to *Colletotrichum acutatum* species complex in the clade A3. From recorded data, we can state that C. *fioriniae* is present in Italy on *C.sativa*, confirming its extreme ability to colonize many cultivated and forest plants. Despite C. *fioriniae* causes anthracnose on different plants (Pszczółkowska *et al*.,2016; Pavel,2016) to date, there is still no correlation between symptoms and the presence of *C.acutatum* on *C.sativa* though it was observed a specific symptom of *Colletotrichum*, called “pink rot “on chestnut nut (Gaffuri *et al*., 2016).

The discovering of this almost worldwide pathogen associated to a new symptomatology on chestnuts confirms a possible risk related to its presence, because the endophytic isolates showed the same pathogenicity of those obtained from infected nuts.

Hence, this fungus might parasitize the larvae and may also be present on galls prior to wasp emergence (Meyer *et al.,* 2015).

Considering the potential pathogenic role of this fungus, many questions still remain unanswered: its presence on healthy chestnut trees opens interesting views on its ecological role and impact both on chestnut ecosystem and on other host.

Introduction

Castanea sativa Mill.

Castanea sativa Mill. belongs to the genus *Castanea,* family *Fagaceae*, and it is the most widespread in Europe while, in Asia, the most present species are *C. crenata* Siebold & Zucc. (Japanese chestnut), *C.mollissima* Blume (Chinese chestnut), *C.davidii* Dode (China) and *C.henryi* (Skan) Rehder & E. H. Wilson (Chinese chinkapin). The American species are C.*dentata* (Marshall) Borkh (American chestnut- Eastern states), *C.Pumila* (L.) Mill*., C.alnifolia* Nutt. (Southern states), *C.ashei* (Sudw.) Sudw. ex Ashe (Southern states), *C.floridana* (Sarg.) Ashe (Southern states) and *C.paucispina* Ashe (Southern states).

C.sativa is a medium large deciduous tree, growing erect and reaching 30-35 m height,firmly set and massive with a columnar trunk, tapering little. The plant is long-living (up to 1 000 years) and may also reaches a significant girth (up to 12 m at breast height) (Conedera *et al.,* 2016). Its bark is brown-grayish with net-shaped venations and deep furrows or fissures. The furrows run longitudinally, but along the years tend to twist, often looking like thick stands associated in a big cable. Leaves are oblong-lanceolate (8-25 cm long, 5-9 cm broad), narrow and glossy and arranged alternately on the twig; the leaf margins have sharp-pointed and distant spreading teeth. They remain on the trees until late in autumn, turning to a golden color. This tree is monoecious: flowers appear in late spring or early summer (in late June to July) and may be pollinated by wind or insects.

Male flowers are gathered in catkins (5 to 15 cm in length) whereas female flowers are usually positioned at the base of the male ones, in the upper part of the current year's shoots. Female flowers develop into spiny cupules (commonly called bur) containing 3-7 brownish nuts (fruits) that are shed during September-October. Some cultivars develop only one large nut per cupule (rarely up to three). The nut is an achene composed of two skins; the external part is shiny brown (pericarp) and the internal is a pellicle adhering to the fruit (episperm), and edible creamy-white cotyledons (Prgomet *et al.,* 2014).

The nuts are roundish in shape, drawn up to a point and flattened on one side, being thus enclosed in a kind of casket protected by spines.

C. sativa: history

The sweet chestnut is an ancient plant, present in the Mediterranean area since prehistoric times, more exactly from the Cenozoic (Kosňovská, 2013).

In the Miocene, the *Castanea* genus was widespread in Europe and was also present in Scandinavia and Greenland as evidenced by the fossil remains of pollen, leaves and fruits (Conedera *et al.,* 2004).

In the last glacial period, the chestnut suffered a remarkable regression but the subsequent climate improvement led to its new expansion. Paleobotanic studies have shown that, in central Italy in 1000 b.C., the chestnut pollen was the 8% of the total one (Conedera *et al.,* 2004); this percentage increased strongly during the expansion period of the Roman Empire, reaching even 48% at the beginning of the Christian era (Conedera *et al.,* 2004).

Even in Latin literature we find numerous details about the origin of *Castanea sativa*: according to Latin literature, chestnut originates from Sardi, city of Lydia (Turkey); Pliny the Elder, in "*Naturalis Historia*", calls it "*nux Castanea*" and lists eighteen varieties, including Tarantina, Balantis, Salariana, Corelliana and Eteniana.

Virgil, who lived in the first century a.C., in the second book of the Georgians suggests grafting the chestnut on the beech, and in the Egloghe mentions chestnuts cooked with milk and eaten with cheese. Martial (1st Century b.C.), in his "*De hortensis*", states that no city could compete with Naples in roasting chestnuts.

Chestnut was widespread during the Roman Empire not only for the cultivation of the fruit but also for the possibility to obtain cedar that was complementary to viticulture.

In the Middle Ages, the work of the monks led to the spread of fruit crops and in the 18th century, woodcutter cultivation was widespread among large landowners. Chestnut became an essential source of food and timber in the Mediterranean and the southern parts of Central Europe (Kosňovská, 2013). In various European regions, particularly in highlands and places without wheat production, *C.sativa* cultivation became predominant and indispensable for the survival of the mountain population and their traditions.

In particular, in Italy, the chestnuts cultivated for nuts were very important and greatly influenced the lifestyle of mountain populations, giving origin to the so-called ‘‘chestnut civilization’’ (Gabrielli, 1994; Arnaud *et al.,* 1997; Conedera *et al.,* 2004). In fact, in some areas chestnut was

called "The tree of bread", as defined by whole generations of mountain populations and it represented for them a source of food otherwise difficult to find.

This medieval golden age of the chestnut declined progressively, however, due to climatic cooling (the Little Ice Age during the eighteenth-century) that caused frost damage on chestnut orchard trees, the introduction of alternative crops from abroad such as maize and potatoes and also due to the Industrial Revolution which brought about higher usage of chestnut trees for charcoal (Kosňovská, 2013). Another cause of the decline of chestnut was a depopulation of the mountain countryside (Bonous, 2001; Conedera and Krebs, 2008) and the appearance and spread of different diseases, in particular: chestnut blight (*Cryphonectria parasitica* Murr. Barr) and ink disease (caused by *Phytophthora cambivora* Petri Buism.) (Vettraino *et al.,* 2005). All these factors had brought to the complete abandonment of traditional chestnut orchards. Nevertheless during, in the first forty years of the twentieth century, chestnut wood was the main outlet for the tannin industry (Kosňovská, 2013).

The disappearance of the traditional chestnut groves represents a cultural as well as an ecological loss that may be considered the consequences of the natural reforestation of abandoned fields and pastures (Pezzi *et al.,* 2011). These changes are so important that they have also been recognized by the European Community Natura 2000 network (EU Council Directive 92/43/EEC), which have declared important habitats (9260 *Castanea sativa* woods) for biodiversity conservation (European Commission 2007) both the *Castanea sativa*-dominated forests and the long-established chestnut plantations with semi-natural undergrowth (Pezzi *et al.,* 2011). In Italy, this directive is very relevant because chestnut woods still dominate the low and middle mountain landscape, both in the Southern Alps and the Apennines.

Changes in land use, environmental diversity, socioeconomic scenarios, and the impact of plant diseases are the potential driving factors for the present species composition in chestnut stands.

C. sativa: habitat and ecology

The sweet chestnut is a warm-temperate deciduous species that likes a mean yearly temperature ranging between 8 ° and 15 °C.

The species needs a minimum rainfall that ranges between 600 and 800 mm (Conedera *et al.,* 2016).

The chestnut tree displays a high sensitivity to summer droughts issuing from the combination of high temperatures and lack of precipitation and it is sensitive to late frost.

It does not thrive on limestone preferring well-drained soils with a pH range between acid and neutral and nutritionally poor site (Conedera *et al.,* 2016). This tree can rejuvenate in half-shadow conditions but needs light for growing from the early pole stage.

C. sativa: distribution

Chestnut tree is economically and environmentally important in Europe and in Italy. It is distributed from Southern Europe (Iberian Peninsula, Italy, Balkans, Mediterranean Islands) and North Africa (Morocco), to North-Western Europe (England, Belgium) and eastward to Western Asia (North East Turkey, Armenia, Georgia, Azerbaijan, Syria) (Conedera *et al.,* 2016), with an altitudinal range between 200 and 1800 m, depending on the latitude and site aspect (Conedera *et al.,* 2016) (Graph 1).

In Europe, the sweet chestnut covers an area of more than 2.5 million hectares. Most of the area is concentrated in France, Italy, Spain, Portugal and Switzerland with a long tradition of chestnut cultivation. Here the widespread distribution of chestnuts, cultivated or naturalized, is crucial for the maintenance of the local biodiversity but also to maintain steep slopes in mountain areas (Quacchia *et al.,* 2008).

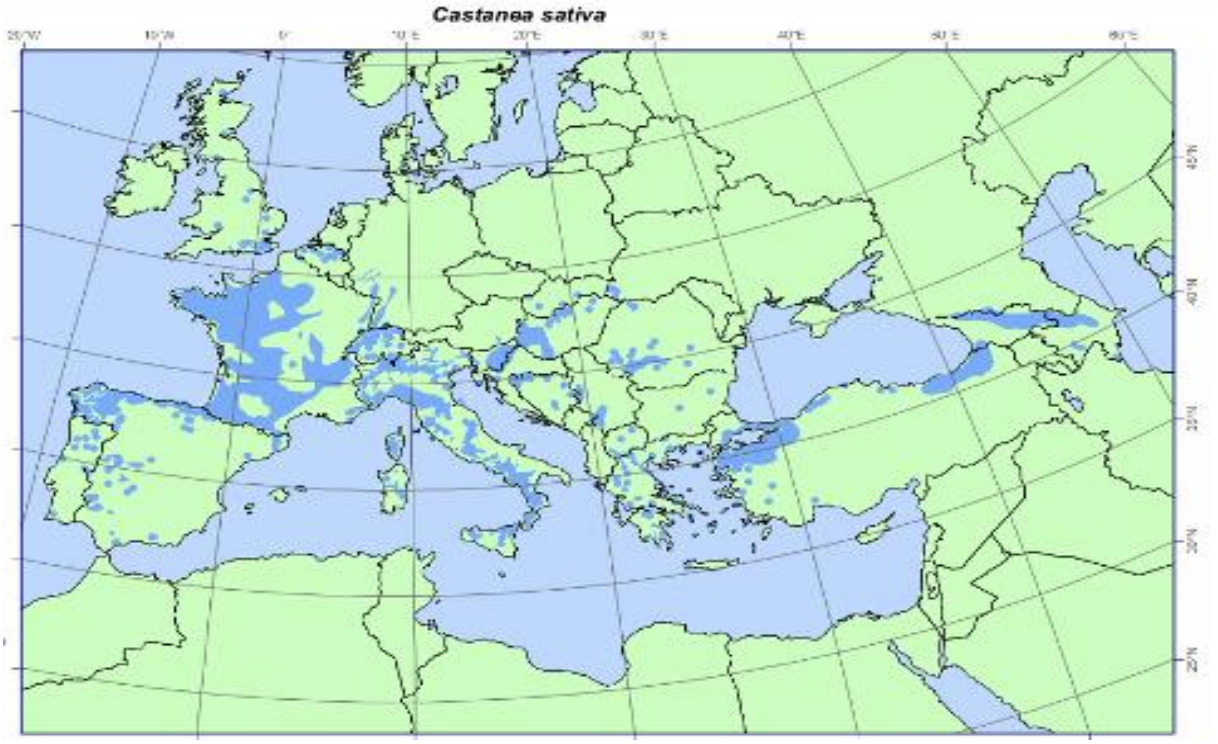
The importance of *C.sativa* can be identified both for fruit production and woods.

World production of nuts is concentrated in two large areas, Asia and Europe, representing respectively 80% and 16% of the world production (FAO, 2008). It should be stressed that the Asian production is obtained from Chinese chestnut species, different from the European ones, with lower organoleptic characteristics. European production has decreased from 11% to 4% due to China increased production that has reached around 170,000 tons.

The main European producers are Italy, Turkey and Portugal, which account for 30%, 29% and 15% of European chestnut respectively.

Italy is the largest exporter of chestnuts for the quality of trade, while it is second for quantity traded; the export price of Italian chestnut is, in fact, higher than that of Asian chestnuts. The ISTAT data show that chestnut production is mainly concentrated in Campania, Calabria, Tuscany, Lazio, Piedmont, Emilia-Romagna and Lombardy.

Regarding chestnut for wood production, in Italy about 10.5 out of 30 total million ha are occupied by forests; overall, over 75% of Italian chestnut is intended for timber production.



Graph 1. Source: EUFORGEN 2009: This distribution map, including both natural and naturalized occurrence, of *Castanea sativa*. Distribution map of Chestnut (*Castanea sativa*) EUFORGEN 2009, www.euforgen.org.

C. sativa: Lombardy distribution

The areas suitable for cultivation of chestnut within each Lombardy province are described below. All the data are from Boriani *et al.,* 2013.

VARESE

The first chestnuts areas in the province of Varese are placed in the west territory of the Ticino Park, north of Sesto Calende, Mercallo, Cuirone di Vergiate and along the mountainous outskirt of Comabbio and Monate lakes.

In the eastern areas, the chestnuts are found in Somma Lombardo, Arsago Seprio and Besnate, along the Pedemontana, in the protected area of the Parco Pineta of Tradate and Appiano Gentile. North of Varese, we find the area of the Castello Cabiaglio, Brinzio, Luino and the slopes of Monte Campo dei Fiori in which the prevalent type of Chestnut groves are the vegetative on loose substrate. Moving forward, the chestnut is located in the forests between 600 and 1200 meters of altitude, on the border with Switzerland.

COMO- LECCO

Chestnut woods are present in the western branch of Como Lake, from Brunate to Bellagio and the forest goes on along Monte Bisbino, Valle D'intelvi and Menaggio. We find *C. sativa* along the Legnone and Pizzo Campanile mountains to Colico. Chestnut is also placed in the Pedemontana area, Parco Spina Verde and in the municipalities of Lipomo, Albese con Cassano and Alzate Brianza.

In the province of Lecco there is the largest wooded area of the Triangolo Lariano, enclosed between the two branches of the lake of Como, that includes the municipalities of Caglio, Asso, Canzo, Oliveto Lario and Valbrona. In the south area, we find chestnuts in Monte Barro Park and along the Valsassina.

BERGAMO

The woods area lies along the reliefs surrounding the municipality of Pontida, Caprino Bergamasco, Palazzago and Valle San Martino.

Proceeding eastwards, we find the Valle Brembana, rich in chestnut forests. Southwest we find the chestnut trees in the municipality of Albino, Pradalunga, Monte Misma and along the Valle del Lujo.

Other chestnuts areas are: Val Cavallina, Casazza and Gaverina Terme. The last settlements of chestnuts are on the border with Brescia along Monte Bronzone in the municipality of Predore.

SONDRIO

Valtellina and Val Chiavenna are the main areas where chestnut trees are present (Photo 1). From east to west, the municipalities concerned are: Dubino, Bioggio, Mello Monastery, Berbenno, Postalesio, Castione Andevenno, Sondrio, Montagna in Valtellina and Val Malenco. The chestnut trees extend then upwards to Tirano and Mazzo di Valtellina.

Finally, *C.sativa* is present from the Spluga Valley to the north of Motta.

Photo 1 (F. Gaffuri): Chestnut groves in Castione Andevenno (Sondrio)

BRESCIA

In the province of Brescia, chestnut trees grow on carbon substrate under unfavorable conditions; they spread equally on the Pedemontana and pre-alpine areas. We find them also in Val Trompia, Villa Carcina and Brione, north of Brozzo and Lodrino.

The trees are commonly located in Monte Orfano, Iseo and Poaveno, north of Maddalena Mountain and in the municipally area close to Gavardo.

Other chestnut trees are present along the Valle Sabbia, Parco Alto Garda, Salò, Toscolano Maderno ,Gargnano and Valle di Vione (Photo 2).

PAVIA

The richest area in chestnut groves in Pavia province is Oltrepò Pavese. Heading up north, the localities of Cà de Bergognoni, Trebbio, Chiusani, Colombara are involved. The chestnut groves are present also in the Staffora Valley, Poggio del Re, Monte dei Marroni, Monte Cucco and Nizza Valley (S. Alberto di Butrio).

Photo 2 (Photo courtesy of M.Boriani): Chestnut groves in Moniga del Bosco - Muscoline (Brescia)

C. sativa: chestnut production in Italy

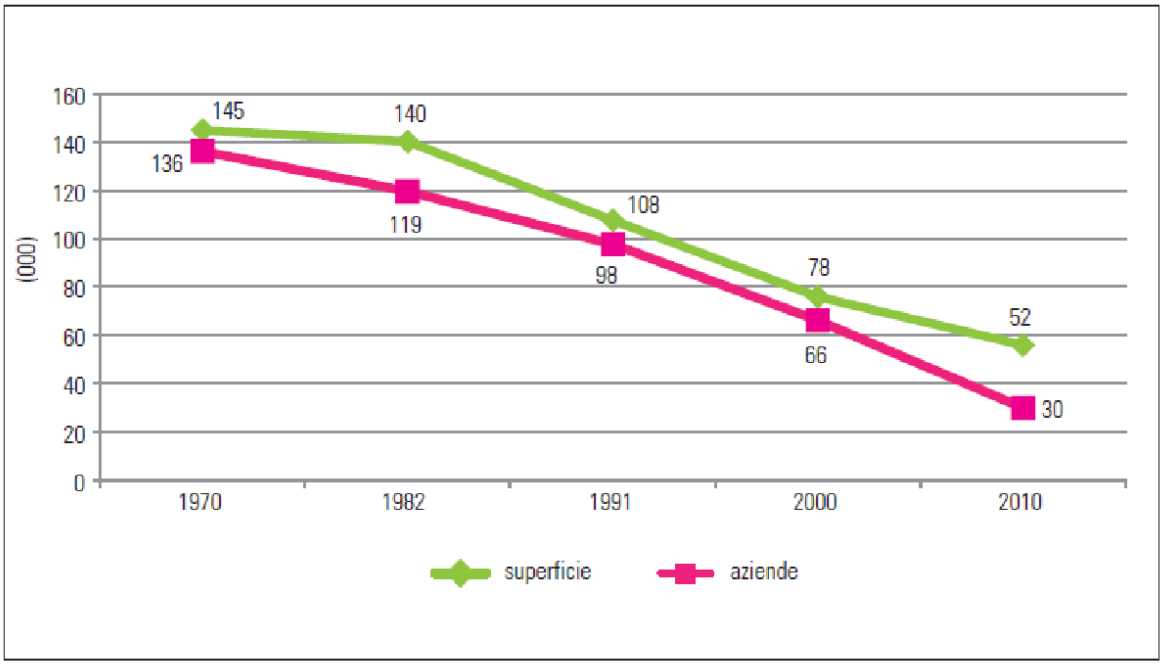
According to the last census data, in Italy farms with chestnut groves for nut-production are about 30,000 on a surface invested equal to 52,000 hectares, 23% of the total utilized agricultural area. The remaining of the surface is invested in meadows and pastures (40%), arable land (23%) and other planting of woodland (14%) (CREA, 2016).

The farms are small-medium size with an average surface area of chestnuts groves of about 2 hectares. This is also due to the depopulation of many mountain areas and unfavorable pedoclimatic environments that have led to prefer permanent grassland and pastures to chestnut cultivation.

Data about the changes of the number of farms and areas invested in chestnuts groves between 1970 and 2010 show a decrease of the areas for nut-production. Between 1970 and 2000, companies have been decreased by 51.3% and the area invested for chestnut by 47.5%, while between 2000 and 2010 the surface area has been reduced from 1.72 to 1.15 hectares. These data show an acceleration in the crisis of Italian chestnut in the last decade (Graph 2).

Observing the surface cultivated with chestnut groves for nut in Italy, five are the regions with more production: Campania, Tuscany, Calabria, Piedmont, Lazio and Emilia-Romagna.

Unfortunately, once again, the regions involved show a reduction in production, number of farms and surfaces (Table 1). In particular, Campania loses 50% of the farms and 13% of the cultivated-areas and, respectively, Tuscany 38% and 36% , Calabria 61% and 39%, Piedmont 43 % and 30% o and Lazio 66% and 32%.



Fonte: ISTAT, Censimento agricoltura, vari anni

Graph 2: Number and surface of Italian farm with chestnut groves for nut production from 1970 to 2010 (Data show ISTAT). Green line shows surface of Italian farm; pink line shows number of Italian farm.

The Italian farms cultivating chestnut are very differentiated as regards environmental, structural, technical and economic matters. Unit yields depend on varieties and their correspondence to the pedoclimatic characteristics and the choice of cultivar is often related to trade issues. The cultivation of varieties of *C.sativa* dominates in Italy by far, but in Piedmont there are many varieties of Euro-Japanese hybrids (Castellotti and Grassi, 2011). Unfortunately, no updated data of Italian chestnuts production are available since the ISTAT no longer detects data through forest statistics. The latest data are related to 2008 (Table 2) and they show that in all regions the traditional extensive farming method, characterized by low density of plantation, low levels of productivity and remuneration, is predominant.

Table 1:

Number of farm with chestnut groves for nut production and surface in the last census data (ISTAT 2010).

Regione	aziende (n.)	superfici (ha)	aziende/Italia (%)	sup./Italia (%)
Campania	6.577	13.808	*22*	*27*
Toscana	5.336	10.399	*18*	*20*
Calabria	4.774	8.643	*16*	*17*
Piemonte	4.052	6.383	*13*	*12*
Lazio	2.063	3.796	*7*	*7*
Emilia-Romagna	1.570	2.822	*5*	*5*
Basilicata	605	1.168	*2*	*2*
Marche	539	838	*2*	*2*
Liguria	823	750	*3*	*1*
Lombardia	785	650	*3*	*1*
Sardegna	304	563	*1*	*1*
Umbria	331	528	*1*	*1*
Sicilia	794	453	*3*	*1*
Veneto	415	359	*1*	*1*
Abruzzo	143	300	*0*	*1*
Trentino-Alto Adige	552	288	*2*	*1*
Puglia	264	122	*1*	*0*
Valle d'Aosta	269	71	*1*	*0*
Friuli Venezia Giulia	40	57	*0*	*0*
Molise	16	4	*0*	*0*
Italia	**30.252**	**52.002**	***100***	***100***

Fonte: ISTAT, VI Censimento agricoltura

Table 2:

Chestnuts production and collection in Italian regions (ISTAT 2004-2008)

Regioni	media 2004-2008 (t)	% sul totale della produzione in quantità	media 2004-2008 (000 €)	% sul totale della produzione in valore	prezzo medio (€/t)	resa media (t/ha)
Calabria*	9.504	*19*	5.694	*10*	599	0,8
Campania	24.873	*49*	26.467	*45*	1.064	1,9
Piemonte	2.024	*4*	1.852	*3*	915	0,4
Liguria**	40	*0*	70	*0*	1.738	0,1
Abruzzo	289	*1*	611	*1*	2.119	1,4
Lazio	7.519	*15*	16.214	*27*	2.156	1,3
Toscana	3.700	*7*	5.221	*9*	1.411	0,4
Emilia-Romagna	634	*1*	1.029	*2*	1.625	0,3
Veneto	80	*0*	179	*0*	2.240	0,4
Lombardia***	547	*1*	765	*1*	1.400	0,5
Sardegna	373	*1*	411	*1*	1.102	0,3
Basilicata	757	*2*	559	*1*	737	1,0
Totale	**50.339**	***100***	**59.072**	***100***	**1.193**	**1,0**

**Media 2004-2007, su dati ISTAT, Statistiche forestali.*

*** Media 2004 e 2008, su dati ISTAT, Statistiche forestali.*

****Media 2004-2006 su dati ISTAT, Statistiche forestali.*

Fonte: Tratto da Castellotti e Grassi (2011)

C. sativa: pests

There are numerous insects that can damage the chestnut (Pollini, 1998), but only a few of them are capable of causing significant economic consequences. Anyway, sweet chestnut is one of the best examples of forest tree species threatened by invasive pathogens and insect pests. A proper management of the stands is necessary to check the different fitosanitary problem.

The main economical damage is usually due to infestations of fruit carried out by *Curculio elephas* Gyll (*Coleoptera*, *Curculionidae*)*, Pammene fasciana* L.*, Cydia fagiglandana* and *Cydia splendana* L. (*Lepidoptera, Tortricidae);* these insect attack the fruits while they are still on the trees, ovideposing their eggs. Usually, damage increases concomitantly with the development of the larvae (Giacalone and Bounous, 1993), able to eat and destroy the nuts. The colonized fruit have no more commercial value. Moreover fungal infection can start in the insect galleries (Wells and Payne, 1980) and, during fruit-storage, degradation occurs (Sieber *et al.,* 2007).

Curculio elephas Gyll

Curculio elephas Gyll spread occurs throughout the distribution of its host *C.sativa* and it is present in oak forests. In fall, the female pierces the chestnut fruit with her long snout and introduces one or several eggs into the nut. The larvae, which are white, with brown head, develop within a month and then chew their way out of the fruit to enter the soil. The following summer, the larvae pupate emerge as adult. The infestation rate of the fruit can be as high as 90% and depends, among other factors, on the length of the sheath spines of the fruit.

Pammene fasciana L.

Pammene fasciana L. is a monovoltin insect, widespread throughout Italy. At the adult stage, the body is along 13-17mm, dark brown coloured, with white wings and three black spot at the sides of the ocular area. The larva is whitish and reaches 13 mm, with a brown stains on the head.

Larvae spend the winter on the cracking of the bark of the tree as cocoon, and remains in diapause until May-June of the following year. Adults appear from June to July and the activity flight is at twilight. The females lay up to 350 eggs on the page upper leaves (Silvestri, 1943).

The larva penetrates in the fruit after crossing the hedgehog and digging a gallery causing darkening of the tissue. On chestnuts, the later attack caused by the larvae determine less damage

than those caused in the first stage. In this case, however, the fruit is damaged not only by larval activity but also by bacteria and fungi that can invade the tissues.

Cydia fagiglandana L.

This *Lepidoptera p*resents adults very similar to the other two with differences only in wings coloration. Its flight occurs in July and August and its behavior is like the other two specise: eggs on burr or leaves, colonization of fruit and , after the fall, exit and overwintering in the soil. Damages are very significant in almost all the stands reaching , with the other two species , almost the 50% of the production in the worse years. The larvae is red.

Cydia splendana L.

Cydia splendana L. is a Lepidoptera with light grey forewings, marbled with light streaks. The distal one-third of the wing is marked with a dark-brown to black sub triangular patch that surrounds the purplish ocellus. Hind wings are dark grayish brown.

Adults may appear similar to those of other *Cydia* species: *Cydia kurokoi* (only located in Asia); *Cydia fagiglandana* and *Cydia pomonella*. Adults are present in Central and Northern Europe during June and July, while in Southern Europe during August and September. Females lay eggs singly on young fruit or on leaves near fruit.

Larvae of *Cydia splendana* are important pests of chestnut. Other preferred hosts include *Quercus, Fagus* and *Juglans*. The damage is caused by the larvae that attack the fruits, the early instars build a tunnel into the fruit and they are fed inside. Usually one single fruit supports one larva. Larval-infested fruit drop to the ground early. Overwintering occurs as a late instar larvae under bark or in the soil.

Dryocosmus kuriphilus Yasumatsu *(Hym., Cynipidae)*

Over the last decades, insect and pathogen invasions have increased exponentially in Europe, damaging forests and other ecosystems (Santini *et al.,* 2013). The introduction of a non-native microorganism may negatively affect the existing tropic interactions altering the equilibrium of natural ecosystems. Plant trade is one of the major pathways of invasion for many fungal pathogens and insect pests (Brasier, 2008).

The chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu (*Hymenoptera, Cynipidae)* is considered one of the most important pests of chestnut worldwide, causing significant damage to European chestnut, as well as to the susceptible Euro-Japanese hybrids (EPPO, 2005). *D. kuriphilus* is a quarantine pest and is included in the A2 list of the European and Mediterranean Plant Protection Organization (EPPO, 2015).

Native to China, it is the only member of the Cynipini tribe which attacks the genus *Castanea*, representing its most significant insect pest globally.

Dryocosmus kuriphilus induces gall formation on petioles and leaves only in *Castanea spp* (Photo 3) and, unlike other *Dryocosmus* species, it is known to induce gall formation on *Quercus* spp as well.

In 2002 *D. kuriphilus* was detected in Italy for the first time (Piedmont, Italy) and, later, was also reported in France and Slovenia (2005), Switzerland (2009), Croatia (2010), Slovakia, Czech Republic (2011) and Spain (Catalonia, 2012) (Melika *et al.,* 2013).

The presence of this insect in Lombardy dates back to 2006, with the first report in Val Seriana (Province of Bergamo), correlated to the irresponsible purchase of infested material in 2004.

Currently the insect is present in all chestnut-groves in Lombardy but the damage has been limited by the Phytosanitary Service (SFR-Lombardy) which, since 2006, has carried out biological controls of the pest, releasing a natural antagonist, the insect *Torymus sinensis* Kamijo.

A first group of the antagonistic insects were released in Albino (district of Bergamo), and the monitoring during the following spring has shown that the population was able to reproduce in natural conditions. Currently, *T. sinensis* is completed naturalized (Boriani *et al.,* 2013).

The biological control, over the years, led to a situation of equilibrium between the reduction of damage and the number of the *D. kuriphilus*.

This approach was the only possible solution, as chemical control would have been unthinkable due to the fact that a chemical product is able to reach the galls and the adults have a scalar flight over time. Therefore, in order to be effective, numerous treatments, facing high costs and compromises about biodiversity have been necessary (Boriani *et al.,* 2013).

Photo 3 (Photo courtesy of G.Maresi): galls produced by *D. kuriphilus* on *Castanea sativa.*

D. Kuriphilus: origin and diffusion

The Asian chestnut gall wasp was reported for the first time in 1941 in Japan (Okayama) and in the following years serious damage to Japanese chestnut orchards was observed (Aebi *et al.,* 2006). In 1958 it was found in Korea, in 1974 for the first time in Georgia (United States) (Aebi *et al.,* 2006) and in 1999, *D. Kuriphilus* was detected in the north of Nepal (Ueno, 2006).

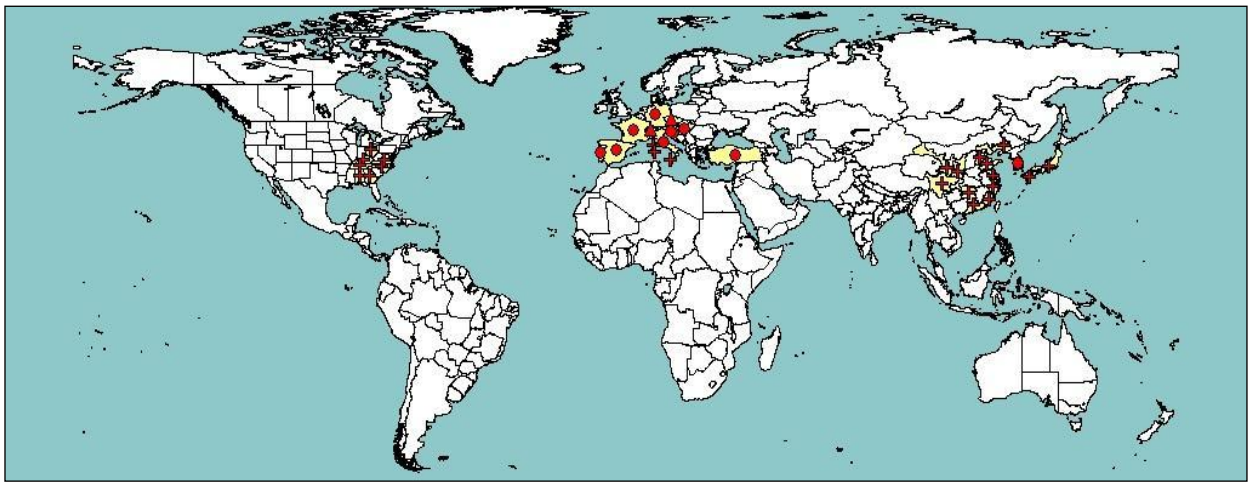
In Europe, it was found for the first time in Italy in 2002 and *s*ubsequently it was reported in France (2005) in an orchard of Saint-Dalmas Valdéblore (Provence-Alpes-Côte d'Azur), in Slovenia (2005) and in chestnut areas of Canton Ticino in Switzerland (2009).

In Italy, *D. kuriphilus* was reported in Cuneo and it is present, under official control, in Abruzzo, Calabria, Campania, Emilia-Romagna, Friuli-Venezia Giulia, Lazio, Liguria, Lombardy, Marche, Toscany, Trentino-Alto Adige, Sardegna, Umbria and Veneto (EFSA, 2010).

The global distribution is shown in Graph 3.

In the first year the local spread of *D. kuriphilus* was mainly supported by the females natural flight;

in the following years transportation by cars and trucks contributed to medium or long distance diffusion such as the movement of young infested plants or scions, where eggs and larvae are undetectable in the buds.



Graph 3: *D. kuriphilus* global distribution (EPPO PQR)

The Chestnut fruit does not represent a spread pathway as no life stage occurs on the fruit and there is no opportunity for contamination from free-living adult stages as they are not present during the fruit harvesting period (EPPO, 2005).

Even the movement of timber and wood packaging material does not represent a pathway due to the absence of bud and leaf tissue, which precludes the presence of immature stages as eggs and larvae.

D. Kuriphilus: identity and morphology

Class: Insecta;

Order: Hymenoptera;

Family: Cynipidae

Genus: *Dryocosmus*

Species: *D. kuriphilus*

Binomial name:

Dryocosmus kuriphilus Yasumatsu, 1951

Photo 4 (Gyorgy Csoka, Hungary Forest Research Institute, Bugwood.org): Adult of *Dryocosmus kuriphilus* Yasumatsu.

The adult female of *D. kuriphilus* (Photo 4) is 2.5–3 mm long on average, the body is black; legs, the scapus and pedicels of the antennae, apex of clypeus and mandibles are yellow brown; the head is finely sculptured; the scutum, mesopleuron and gaster are highly polished and smooth; the propodeum has 3 distinct longitudinal carinae; propodeum, pronotum are strongly sculptured; the scutum has 2 uniformly impressed and pitted grooves (notaulices) which converge posteriorly; the radial cell of the forewing is open; the antennae 14 segmented with apical segments are not expanded into a club (Bosio *et al.,*2010).

The female lays eggs into the buds of current shoots in June and July. The eggs are oval, milky white, 0.1-0.2 mm long, with a long stalk. The growth of *D. kuriphilus* occurs first as a larva and then as a pupa: the larva is 2.5 mm long when fully grown, milky white, without eyes and legs; the pupa is 2.5 mm long, black or dark brown.

D. Kuriphilus: life cycle

Dryocosmus kuriphilus is a univoltine species that reproduce parthenogenetically and is the only species able of modifying the chestnut buds turning them into galls differentiating them from all other all wasp insects mainly linked to oaks.

This insect lives exclusively on *Castanea*; in Italy it has been reported on hybrids (Euro-Japanese) and on European chestnut (Bernardo *et al.,* 2013); in particular, it attacks *Castanea crenata*

(Japanese chestnut), *Castanea dentata* (American chestnut), *Castanea mollissima* (Chinese chestnut) and *Castanea sativa* (European chestnut). In China, *D. kuriphilus* infests also *Castanea seguinii* (Bosio *et al.,* 2005).

Populations of *D. kuriphilus* are composed entirely of females (Bernardo *et al.,* 2013) and they are short-lived (2-10 days) (Yasumatsu, 1951).

Each female can lay more than 100 eggs during a lifespan (EPPO, 2005) but on chestnut gems they are able to lay groups of 3 to 5 eggs. The larvae emerge from the eggs at 30–40 days and overwinter in quiescence (EPPO, 2005). Adults emerge from the galls depending on locality (altitude, exposure) and chestnut cultivar; in the Lombardy area this occurs during the last week of May and early June (Brussino *et al.,* 2002).

After the adult emergence, the gall dries, becomes wood-like and remains attached to the tree up to two years.

D. kuriphilus: symptoms and damage

Galls are unilocular or multilocular, depending on the development of the insect present on young twigs, on leaf petioles or on leaves, they measure 5-20 mm in diameter and are green or rose-colour. The galls are readily detected on plants or part of plants, while eggs and first instars larvae inside the buds cannot be detected by simple visual inspections.

After adult emergence, the gall dries, becomes wood-like, and remains attached to the tree up to two years.

The creation of galls results in preventing or inhibiting the development of normal shoots and the production of abnormal plant structures (Maltoni *et al.,* 2012) (Photo 5A-B). This leads to: (*a*) a progressive loss of the photosynthetic biomass (Kato and Hijii, 1997), (*b*) a decrease in tree vigor (Kato and Hijii, 1997) and (*c*) an increase in branch mortality due to gall wasp post-emergence fungal attacks (Meyer *et al.,* 2015).

In cases of severe *D. kuriphilus* attacks, mortality of young plant or weak plants occurs, strongly reducing the quality and quantity of timber (Kato and Hijii, 1997; Maltoni *et al.,* 2012), nuts and flowers.

The formation of galls, determined by *D. kuriphilus*, disrupts twig growth and reduces fruiting, causing, in some provinces, yield reductions of up to 60-80%. This reduction is similar to that

recorded in other countries (Dixon *et al.,* 1986); nevertheless also the climatic trends, with long dry seasons and often with high temperatures, have led to the reduction in yield.

A) B)

Photo 5 (F.Gaffuri): A) Gall produced by *Dryocosmus kuriphilus on Castanea sativa* B) Longitudinal section of the gall, presence of *D.kuriphilus* larvae.

D. kuriphilus: phytosanitary measures

Since 2003, after the agreement of a specific Pest Risk Assessment (PRA), *D. kuriphilus* has been inserted to the EPPO A2 Action List, and has been regulated as a quarantine pest.

Imports of all plants of *Castanea* (except fruits and seeds) from non-European countries are forbidden and production of the plants for *Castanea* planting (young plants or shoots for grafting) within the EPPO region should be produced in a place kept free from *D. kuriphilus*.

Currently, in Europe, there are other regulations governing the control of the insect, in particular:

A. Commission Decision of 27 June 2006 (2006/464/CE) "On provisional emergency measures to prevent the introduction into and the spread within the Community of *Dryocosmus kuriphilus* Yasumatsu".
B. D.M 30 October 2007 transposition of Commission Decision "Misure d'emergenza provvisorie per impedire la diffusione del Cinipide del Castagno, *Dryocosmus kuriphilus* Yasumatsu, nel territorio della Repubblica italiana".
C. In Lombardy, the legislation n°10528, 21 November 2012 is in force : "Nuove misure fitosanitarie obbligatorie contro il Cinipide del Castagno *Dryocosmus kuriphilus* Yasumatsu in Lombardia" published on 26 November 2012.

Torymus sinensis Kamijo

Dryocosmus kuriphilus Yasumatsu does not represent a threat in its native country and this suggests that in China insect populations are controlled by natural enemies. Unfortunately, when *D. kuriphilus* was accidentally introduced in Italy, no competitors were present in its niche and, for this reason, it was able to proliferate.

The main parasitoid associated with the Asian chestnut gall wasp is *Torymus sinensis* Kamijo. It was released as a biocontrol agent in Japan in 1975, in the USA in 1977 (Picciau *et al.,* 2017) and in 2002 in Europe (Italy) (Brussino *et al.,* 2002).

The introduction of this parasitoid has been a biological success in chestnut-cultivated areas in Italy, gradually reducing *D. kuriphilus* epidemic (Ferracini *et al.,* 2015).

Torymus sinensis Kamijo: identity and morphology

Class: Insecta;

Order: Hymenoptera;

Family: Torymidae

Genus: Torymus

Species: *T. sinensis*

Binomial name: *Torymus sinensis* Kamijo

Photo 6 (Photo courtesy of S.Sacchi):Torymus sinensis.

Torymus sinensis (Photo 6) belongs to the superfamily Chalcidoidea (Murakami *et al.,* 1977); it is a univolt species (Picciau *et al.,* 2017) of approximately 2.5mm in length, it has one generation per year with an average life of about 25-30 days, and it consists of female and male individuals .

Its body is black with legs, while the scapus and pedicels of the antennae, the apex of the clypeus while mandibles are yellow brown; its head is finely sculptured; the scutum, mesopleuron and gaster are highly polished and smooth; the propodeum has three distinct longitudinal carinae; propodeum and pronotum are strongly sculptured (Murakami, 1981). Males differ from females because of their blackish scape with metallic reflections and the tibiae are extensively darker, usually with metallic reflections (Kamijo, 1982), while the female have the ovipositor.

T. sinensis: biological control

The Japanese experience has been a precursor for many realities in Italy, in fact biological control against *D. kuriphilus* was accomplished by releasing *Torymus sinensis* into infested sites. This method is called “Propagation method”.

The parasite, in the winter time, grows in the galls to become adult in spring and to continue another biological cycle (Quacchia *et al.*, 2011).

This protocol for the biological control rises from the experience developed by the DIVAPRA, a research institute of the University of Turin.

In order to achieve this goal, some couples of *T. sinensis* obtained from parasitized galls collected in “areas of multiplication” were released in open fields .

“Areas of multiplication” were created in chestnut orchards to obtain good amount of *T. sinensis* in about 3 - 4 years.

C.sativa: diseases

Climate change and declining markets, together with biotic factors have influenced Chestnut culture. The main pathogens that have been potential threats to the decline of chestnut throughout Europe are: *Cryphonectria parasitica*, causing chestnut blight and *Phytophthora cambivora* and *Phytophthora cinnamomi*, the ink disease agents (Bounous, 2001). Other damage on leaves, when weather conditions are favorable, we can find *Mycosphaerella maculiformis*, agent of "fersa or fog" and *Microsphaera quercina* cause of "powdery mildew"; on the fruits ubiquitous or parasitic fungi can be identified, sometimes able to produce severe damages. Recently, the presence of *Colletotrichum* on leaves, branches and galls of *D. kuriphilus* was reported for the first time in Italy and Europe (Gaffuri *et al.,* 2015). This organism does not appear to cause damage to the tree of *Castanea sativa*, but has been identified on the fruit, for the first time, associated to the symptom called "Pink rot" (Gaffuri *et al.,* 2016).

"Ink disease" caused by *Phytophthora spp*.

"Ink disease" was considered the primary threat to chestnut survival in Europe. It was recorded in Portugal in 1838 for the first time, after that it diffused in Europe epidemically.

The presence of the disease in Italy was reported in 1875 (Gibelli, 1879) and its cause was ascertained in 1917 (Petri, 1917).

In the literature two species are considered being responsible for "Ink disease" of sweet chestnut (*Castanea sativa* Mill.) in Europe: *P. cambivora* (Petri) Buis and the more aggressive *P. cinnamomi* Rand (Petri, 1917; Milburn and Gravatt, 1932; Crandall *et al.,* 1945; Grente, 1961).

However, some other *Phytophthora* species, such as *P. citricola* Sawada*, P. cactorum* (Lebert and Cohn) J. Schröt., *P. cryptogea* Pethybridge & Lafferty *P. gonapodyides* (Petersen) Buisman and *P. pseudosyringae* T. Jung & Delatour cause the disease of sweet chestnut (Erwin and Ribeiro, 1996; Vannini and Vettraino, 2001; Vettraino *et al.,* 2001; Scanu *et al.,* 2010), but their impact is considerably lower.

In the nineties, the unaccustomed resurgence of "Ink disease" was observed especially in Portugal, Italy and France, limiting the establishment of new groves and the conservation of the old ones (Vettraino *et al.,* 2001). In the last decade, the sweet chestnut population in Portugal has

decreased because new plantations have not been sufficient to exceed the number of dead chestnut trees, killed mostly by “ink disease” (Martins *et al.,* 2007).

P. cinnamomi and *P. cambivora* are distributed from Greece to Great Britain. To date, the greatest impact of the disease is limited to the southwest, southern and warmer regions of central Europe (Brasier and Jung, 2005; Erwin and Ribeiro, 1996; Juhásová, 1999; Oszako *et al.,* 2005; Vannini and Vettraino, 2001; Vettraino *et al.,* 2005; Werres *et al.,* 2001) and in Czech Republic (Černý *et al.,* 2008).

Symptoms of the disease on adult trees include chlorotic leaves reduced in size, thinning of the crown, and immature husks remaining on the tree after leaf-fall.

Flame shaped dark necrosis are evident on the collar of the tree after debarking. The large roots are the mainly infected. They produce a black exudates that stains the surrounding soil, especially during spring and fall. On young trees with smooth bark, the necrosis are visible without debarking as depressed, slightly cracked areas at the base of the stem (Vannini *et al.,* 2011) (Photo 7). Infected seedlings in nurseries or plantations undergo a rapid or gradual wilting of the leaves. In the root system, there is extensive necrosis of the tap root that extends to the lateral roots and up the stem for some centimeters (Vannini and Vettraino, 2001).Beneath the bark, at the base of the tree, infected tissues appear depressed with small cracks and callus reactions at the margins; dark necrosis can be observed on stem and roots.

At the time the symptoms are visible, the tree may have been infected for several years. The infected trees are killed within 2-3 seasons. The spread of disease can occur through flowing water. Moreover, human activities such as hunting, mushroom harvesting, and some animals, such as wild boar and field-mice, can likewise be considered passive vectors of the disease control of the disease in very difficult and it is tried both with organic manuring on slightly affected plants or by chemical injection (Turchetti and Maresi 2008; Vannini and Vettraino, 2001).

Photo 7: (Photo courtesy of A.Tantardini): Symptoms of “Ink disease” which infects *Castanea* tree root.

"Chestnut blight" caused by *Cryphonectria parasitica* (Murrill) M.E. Barr

Cryphonectria parasitica (Murr.) Barr. (Syn. *Endothia parasitica* (Murril) P. J. Anderson & H. W. Anderson) (anamorph: *Endothiella*) (Photo 8) is the causal agent of "chestnut blight" disease which infects the tree species of *Castanea* and *Quercus* genera. Taxonomically, *C.parasitica* is included in *Ascomycota phylum*, *Ascomycetes* class, *Sordariomycetidae* subclass, *Diaporthales* order, *Valsaceae* family and *Cryphonectria* genus by Kirk *et al.,* (2001). Recently a new family Cryphonectriaceae was proposed in the Diaporthales family including *Cryphonectria* genus, by Gryzenhout *et al.,* (2006). The chestnut blight fungus was first described in 1906 as *Diaporthe parasitica* Murr. and renamed in 1912 as *Endothia parasitica* (Murril) P. J. Anderson & H. W. Anderson (Griffin and Elkins, 1986).

Originally a weak pathogen on Chinese chestnut, the fungus was first observed in North America, at the beginning of the 19th century, when American chestnut trees (*Castanea dentata* [Marsh.] Borkh) started to die in the New York Zoological Garden (Anagnostakis, 1987). The disease spread in European chestnut stands, following the first identification in northern Italy in 1938 (Biraghi, 1946). the impact of this invasive pathogen was dramatic in America where in forty years it destroyed almost all the American chestnut. At the beginning of its appearance in Europe severe damages were observed also on European chestnut and in the 50th's its pread in the italian stands probably increased the abandonment of the cultivation.

As classic parasite of wounds, *C.parasitica* penetrates through bark lesions caused by hailstones, natural bark cracks, cuts made through pruning and grafting (Robin *et al.,* 2001).

The colonization of bark tissues causes the appearance of reddening and depressed bark, and, when the infection girdles the affected branch or stem, a sudden dead of the above part follows, releasing dead leaves. On the cankers several fruiting bodies (pycnidia and perithecia) develops and spores are spread by rain, insects, snails and birdsas like as human beings. In Europe, chestnut tree is the main host of *C. parasitica*, though the fungus can infect oak trees (*Quercus*), on which it only causes slight and rare damage (Turchetti and Maresi , 2008; Robin *et al.,* 2001).

The disastrous spread of the parasite on European chestnut was naturally checked by the appearance of hipovirulence, first detected in 1952 by Biraghi in Italy (Turchetti and Maresi, 2008). Infection doesn't develops in normal canker as describe above but colonizes only the external tissues of the bark, without killing the cambium. So the affected plant is able to survive and

continues to vegetate without other symptom than these abnormal infection called healing and healed cankers. Nowadays, the natural spread of this phenomenon, due to the presence in the population of the fungus of viral particle called dsRNA (Choi and Nuss, 1992) and enhanced by other not already well understood ecological and biological factors (Turchetti and Maresi , 2008), is causing the recovering and the survival of most of the affected chestnut stands. Predominance of abnormal infection and the consequent strong reduction of the damages is occurring almost everywhere in Italy and in most of the other European countries (Heiniger and Rigling,1994; Turchetti and Maresi, 2008)

A) B)

Photo 8 A-B (Photo courtesy of A.Tantardini): Symptoms of “chestnut blight” disease on *Castanea sativa*: healing (B) and healed (A) infections.

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C.sativa: nuts diseases

The cultivation of chestnut has been rediscovered by consumers and producer for the nutritional value of nuts. Increasing numbers of producer associations forming to promote the cultivation of sweet chestnuts and the production of traditional nuts, based food of commodities, have been observed (Dennert *et al.,* 2015).

Market-level quality of nuts is difficult to obtain because disease prevention strategies are limited and pesticide and fungicide use is permitted only in sweet chestnut orchards and forests under exceptional concessions.

There are several fungi causing nut rot at pre- and/or post-harvest, resulting in yield and economic losses (Lione *et al.,* 2015): *Phoma endogena* Speg. and *Phomopsis endogena* Speg. have been described as agents of "brown rot", *Sclerotinia pseudotuberosa* Rehm (syn. Ciboria batschiana Zopf) can cause "black rot", while *Penicillium* spp. and *Penicillium crustaceum* L. Fr. produce greenish moulds and G*nomoniopsis castaneae* is the causal agent of "brown rot" (Visentin *et al*., 2012.

G. castanea is an emergent nut rot agent present in several areas of Europe (Visentin *et al*, 2012; Maresi *et al.,* 2013 the symptoms include a chalky aspect of the nut kernel at ripening, turning to brown as the mummification advances and the mycelium occupies the kernel tissues (Maresi *et al.,* 2013).

Visentint *et al.* (2012) demonstratied that the fungus was an endophyte and following works confirms the presence of the fungus directly from female and male flowers, mature burrs, leaves, kernels and in wood and bark (Shuttleworth *et al.,* 2013, Vannini *et al.,* 2014).

Gnomoniopsis sp. was found in *D. kuriphilus* galls (Magro *et al.,* 2010) where causes necrosis and in some cases the insect death (Magro *et al.,* 2010; Vannini *et al.,* 2011).

Some studies report that the incremental of the *G. castanea* brown rot follows the gall wasp infestation in Italy, and speculate an existing interaction between the two organisms (Turchetti *et al.,* 2012; Maresi *et al.,* 2013). Moreover, Vannini *et al.,* (2017) show that isolation of *G. castanea* is more successful from the asymptomatic tissues sampling from trees that have been heavily infested by gall wasp.

Till now the spread of this problem seem related to the insect presence(Lione *et al.*, 2016) and to climatic factors (Lione *et al.*, 2015).

Colletotrichum

Colletotrichum is a genus of filamentous fungus recently voted the eighth most important group of plant pathogenic fungi in the world, based on perceived scientific and economic importance (Dean *et al.,* 2012).

Virtually every crop grown throughout the world is susceptible to one or more species of *Colletotrichum*. These fungi cause anthracnose spots and blights of aerial plant parts and post-harvest rots. The damage caused by *Colletotrichum* spp. extends to important food crops, including bananas, cassava and sorghum, grown by subsistence farmers in developing countries throughout the tropics and subtropics. It is particularly successful as a post-harvest pathogen because latent infections, which are initiated before harvest, do not become active until the fruit has been stored or appears on the market shelf. Up to 100% of the stored fruit can be lost as result of *Colletotrichum* disease (Prusky, 1996; Wonsu *et al.,* 2016).

Anthracnose is a general term used to describe diseases that result in a wide range of symptoms including leaf spots, blotches or distortion, defoliation, shoot blight, twig cankers and dieback on many different deciduous trees and shrubs.

The disease produces great economic losses, estimated above 50% in crops of tamarillo, mango, blackberry, passion fruits (Afanador *et al.,* 2003), strawberry, cranberry, olives and other cultivated perennials in tropical regions (Onofre and Antoniazzi, 2014; Waller, 1992) and ornamental plant in different countries (Reed *et al.,* 1996; Wade *et al.,* 2001).

In general, anthracnose symptoms include dark necrotic lesions, which are oval or angular; plant can be superficially affected during all the stages of maturation (from seedlings to mature plants). Depending on the species of plant affected, different symptoms are observed and identified with a specific name . For example, on strawberry, *Colletotrichum* causes “black spot” on fruit but attacks also roots and leaves (Freeman *et al.,* 1997), on olive tree, it causes anthracnose and is the most important fungal disease that causes chlorosis and necrosis of the leaves, defoliation, and dieback of twigs and branches (Graniti *et al.,* 1993; Prota, 1995).

Taxonomy and nomenclature in the group is confused, even between scientists working in the field, and inaccurate diagnosis of species is not uncommon.

The genus *Vermicularia* (Tode, 1790) could be regarded as an earlier name for *Colletotrichum* according to some interpretations of the Code of Nomenclature for Algae, Fungi and Plants.

Thus the name *Vermicularia* has been adopted quite widely for curved-spored species, even though the type species of *Colletotrichum* has the conidia curved. The genus *Gloeosporium* (Montagne 1849) was also frequently confused with *Colletotrichum* in the late 19th and early 20th centuries. It was used for taxa of *Colletotrichum* without conidiomata setae (their development in many species is variable) but also included quite unrelated fungi.

The generic name *Colletotrichum* was introduced by Corda for *C.lineola*, a species associated with a member of the *Apiaceae* in the Czech Republic. *Colletotrichum lineola* was long considered a synonym of the older taxon *C.dematium*, but has been recently re-established as an independent species (Damm *et al.,* 2009). During the 19th and early 20th centuries, both names *Colletotrichum* and *Vermicularia* were used for several species. More than 10 generic synonyms for *Colletotrichum* were listed by Sutton in 1980 and in 1992 the name *Colletotrichum* was established, as reported (Sutton, 1992).

In the first formal monographic treatment of *Colletotrichum* (von Arx, 1957), based on morphological characteristics with little or no emphasis on pathological features, around 750 names were in existence. Afterwards, the accepted species were reduced from 750 to 11, within a total of 23 accepted specific and infraspecific taxa. In 2009 (Hyde *et al.,* 2009 a) a total of 66 species were accepted, with 20 names considered as doubtful. This assessment represents a substantial increasement in the number of recognised species compared to the 23 taxa identified by von Arx (1957) and the 39 species accepted by Sutton (1992); this reflects the increasing reliance on molecular methods for species definition. In 2012 Cannon *et al.,* showed that further 41 species were introduced, bringing the current number of accepted *Colletotrichum* species over to 100. In that review the taxonomic placement of the genus is discussed, the evolution of their approach to species concepts and anamorph-teleomorph relationships is described, and the history of classification is reported.

Other important contributions were given by Simmonds (1965), who first described *Colletotrichum acutatum* J.H. as a separate species (Position in classification: *Glomerellaceae, Glomerellales, Hypocreomyc*et*idae, Sordariomyc*et*es, Pezizomycotina, Ascomycota,* Fungi), and by Sutton (1980), who accepted 22 species belonging to the genus. In this study, Sutton focused mainly on morphological and cultural characteristics, and most of the taxa were considered plurivorous. Similar approaches were adopted by Smith & Black (1990) for strawberry species and by Walker *et*

al., (1991) for the species associated with *Xanthium*, but with greater emphasis on the integration of taxonomic and pathological data.

From the 90's, with the introduction of molecular study, Mills *et al.,* (1992) and Sreenivasaprasad *et al.,* (1996), identified sequence variations in the ITS1 region of nrDNA between six species of *Colletotrichum*, as well as detecting polymorphisms in the same region between *C. gloeosporioides* strains from different hosts.

In 1994 (Sherriff *et al.,* 1994), the first bootstrapped NJ trees for *Colletotrichum* was presented using ITS2 and LSU sequences of 27 strains indicated as belonging to 13 species; in 1996 Sreenivasaprasad *et al.,* (1996) published a second phylogenetic study of the genus using parsimony analysis of ITS1 and ITS2 sequences from 18 species of *Colletotrichum*, and the authors were able to identify six infrageneric groups.

The molecular analysis took over and was completely indispensable for the determination of *Colletotrichum* genus.

High number of articles were published based on the genetic and phylogenetic analysis associated with a particular crop. The first multilocus phylogenetic analyses of *Colletotrichum* species was published by Talhinhas *et al., (*2002) and in the following years Guerber *et al.,* (2003) introduced glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glutamine synthetase (GS) nucleotide sequences for the study of the *C.acutatum* group.

Colletotrichum acutatum J.H. Simmonds

Teleomorph: *Glomerella acutata* Guerber & J.C.Correll

Colletotrichum acutatum is an Ascomycete belonging to the class Sordariomycetes, family *Glomerellaceae;* originally identified by Simmonds in 1965, described from diseased tissues of *Carica papaya, Capsicum frutescens* and *Delphinium ajacis* in Australia and validated in 1968. Disseminated infections on sea turtles (Manire *et al.,*2002) and several insects (Marcelino *et al.,* 2008) were reported.

In Peres *et al.,* (2005), *C.acutatum* lifestyles are reported. The fungus is a common pathogen of a wide array of crops and non-cultivated plant species (Adaskaveg and Hartin, 1997; Afanador-Kafuri *et al.,* 2003, Arauz, 2000; Bernstein *et al.,* 1995; Dingley and Gilmore, 1972; Freeman and Katan,1997; Maas and Howard, 1985; Peres *et al.,* 2002, Timmer *et al.,* 1994; Wharton and Diéguez-Uribeondo, 2004). It is cosmopolitan in its distribution and it causes extensive crop losses every year. Disease symptoms range from fruit, rots, shoot, leaf and flower blights. Common hosts include many dicotyledonous plants such as strawberry, apple, citrus and stone fruits, but serious diseases on leather leaf fern and pines also have been reported (Dingley and Gilmore, 1972; Norman and Strandberg, 1997).

C.acutatum, for many years, has been regulated as plant quarantine pests by EPPO (European and Mediterranean Plant Protection Organization) even if it is absent from the current list (EPPO 2011), probably because of its widespread distribution in Europe. Many are the studies on *C. acutatum* on morphological and genetic characteristics, particularly in relation to *Colletotrichum gloeosporioides* because they are morphologically similar and have an overlapping host range. Now, these species are considered as complexes due to genetic differences. In 2002, Vinnere hypothesized the possibility of further divide *C.acutatum* into two groups (Vinnere *et al.,* 2002): *C. acutatum* sensu lato and *C.acutatum* sensu strictu. Eight groups were also established (A1-A8) based on rDNA ITS and beta-tubulin DNA (TUB) sequences (Talhinhas et Within *al.*, 2005) but Whitelaw-Weckert *et al.,* (2007) recognised an additional group A9.

In particular, Shivas & Tan (2009) recognized three distinct groups within australian *C.acutatum* strains and accepted two new species, *C.simmondsii* and *C.fioriniae* (formerly *C.acutatum* f. sp. *fioriniae*) as groups A2 and A3 respectively, and more recently, the new species C.clavatum was described as group A4 (Faedda *et al.,* 2011; Damm *et al.,* 2012).

Colletotrichum acutatum sensu lato

C. acutatum sensu lato can be considered a group of species with morphological and genetic diversity (Guerber *et al.,* 2003) and the relationships among populations from different host plants are not yet clear (Freeman *et al.,* 2001; Guerber *et al.,* 2003; Lardner *et al.,* 1999).

Thus the concept of *C.acutatum* sensu lato has been introduced to accommodate the isolates that cluster with *C.acutatum* but diverge from other species of *Colletotrichum* based on molecular criteria (Johnston and Jones, 1997).

The current discussion over classifying this pathogen as a single heterogeneous species or multiple species, focuses mainly on whether genetic differences are sufficient to delineate species in the absence of additional morphological or physiological evidence (Hyde *et al.,* 2009; KoKo *et al.,* 2011). In 2005, eight *C.acutatum* sensu lato subgroups were delineated basing the identification on analysis of ITS sequences (Sreenivasaprasad and Talhinhas, 2005) and, more recently, the group was disaggregated into 31 subgroups based on phylogenetic analysis of six genes and morphological evidences (Damm *et al.,* 2012).

Morphologically *C. acutatum* sensu lato has typical conidia aseptate, hyaline, straight and smooth, fusiform to cylindrical, with one or two acute ends. Conidia sizes vary considerably between different species of the *Colletotrichum*: length × width (µm ^{2}) varied from 6.7 × 4.1 µm ^{2} to 22.3 × 4.5 µm ^{2} (Damm *et al.,* 2012). Conidiophores are hyaline, smooth-walled and very simple. On the plant host, conidia are produced in acervuli as pink or orange masses. Setae are rarely observed; if present, they are dark brown (Lenné *et al.,* 1984). Appressoria are solitary, light to medium brown, smooth-walled, clavate to obovate and borne on undifferentiated hyphae. Colonies on potato dextrose agar (PDA) are white, pink orange in color, and will turn gray or black over time (Damm *et al.,* 2012).

Colletotrichum: Phylogenetic analysis

The continuous reestablishment of the genus and the substantial increase in the number of recognized species is an indication of the complexity of *Colletotrichum* group. Information about *Colletotrichum* species have not always been completed, creating often misidentification, misapplication of names and different species concepts. Many errors were created by the association of the fungus to the plant host species. It was wrongly expected that all the species are host-specific and that only one species of *Colletotrichum* can parasitise each host of one genus; moreover, the introduction of molecular methods raised problems initially.

The first molecular analysis were based on ITS sequence that proved unsatisfactory for species definition because the gene is too evolutionarily conservative to distinguish between taxa (Crouch *et al.,* 2009). For this reason many other molecular markers have a better diagnostic potential for the Fungi, including most of those which are currently used for phylogenetic analysis of *Colletotrichum*; in particular the genes are TUB and TEF1 (James *et al.,* 2006), MCM7 and Tsr1 (Aguileta *et al.,* 2008). Subsequently, for multilocus analysis, other diagnostic markers have been identified, such as GAPDH, which resolved all 29 subclades (Cannon *et al.,* 2012).

Multilocus molecular phylogenetic analysis (ITS, ACT, TUB, CHS-1, GAPDH, HIS3) of 331 strains previously identified as *C.acutatum* and other related taxa, including strains from numerous hosts with wide geographic distributions, confirmed the molecular groups previously recognized and identified a series of novel taxa.

The current phylogenetic analysis reveals that the genus *Colletotrichum* includes nine major clades, as well as a number of small clusters and isolated species.

Multilocus analysis of *C. acutatum* clade show that *Colletotrichum acutatum* is closely related to 29 species, with *C. orchidophilum* as sister taxon. There were some attempts to address some species via adoption of *formae speciales e.g. C. acutatum f.* sp. *pineum* (Dingley & Gilmour 1972), *C. acutatum f.* sp. *hakeae* (Lubbe *et al.,* 2004) and *C.acutatum f.* sp. *fioriniae* (Marcelino *et al.,* 2008), but this mechanism for recognition of pathology-related taxa is now rarely used.

At this point, it is widely presumed that *C. acutatum* is a species complex containing a number of constituent taxa, but there is substantial reluctance to recognise the clades involved as independent species due to the lack of different morphological and cultural characters. For

example, *C. lupini* was not recognised as formally separate from *C. acutatum* by Talhinhas *et al.,*(2002) or by Sreenivasaprasad & Talhinhas (2005).

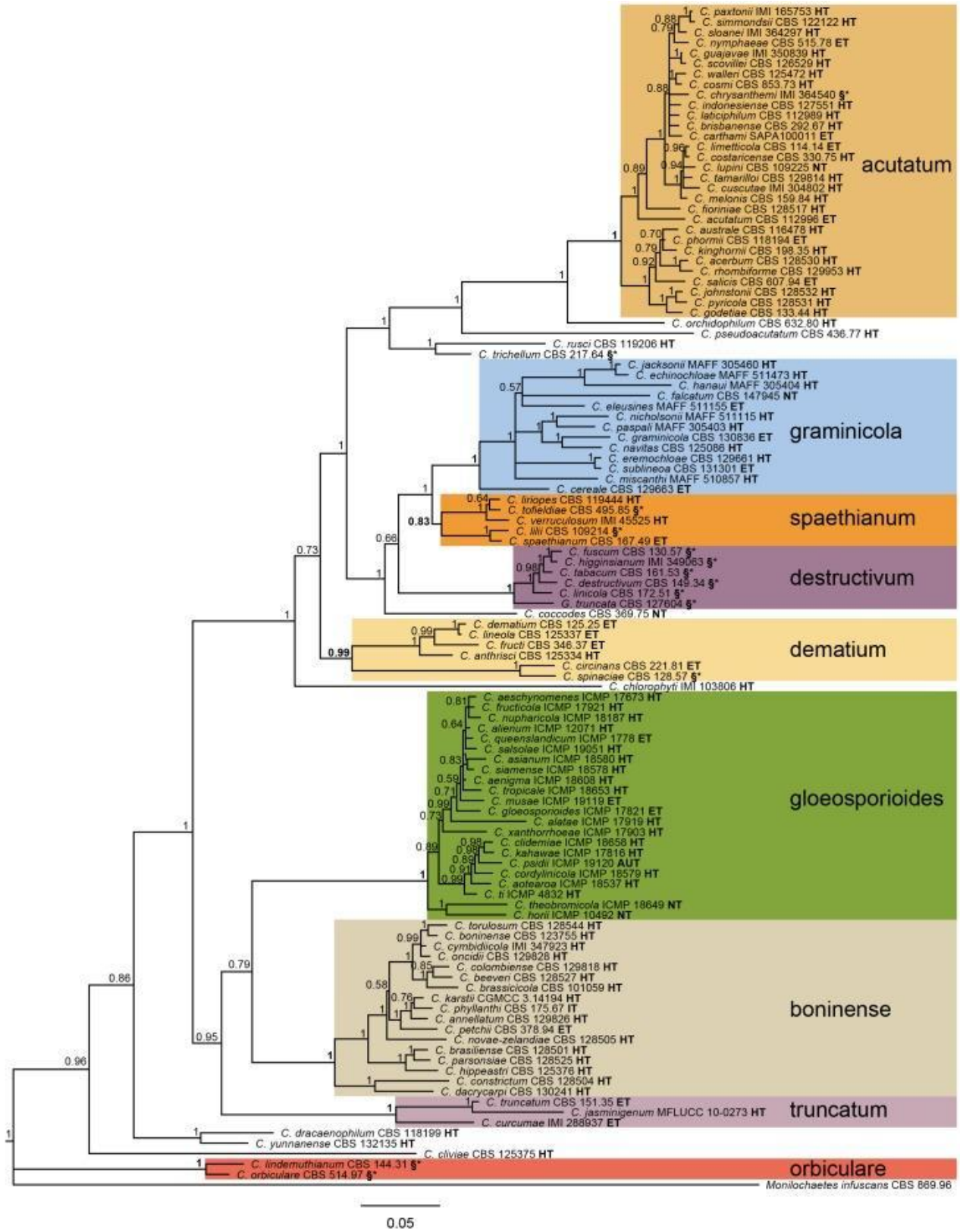

Figure 1 (Cannon *et al.,* 2012): Phylogenetic tree derived from a Bayesian analysis of a partitioned, concatenated alignment of CHS-1 (251 bp), ACT (305 bp), TUB (545 bp) and ITS (599 bp) sequences.

Colletotrichum fioriniae

Glomerella fioriniae (Marcelino & Gouli)

Glomerella acutata var. fioriniae (Marcelino & Gouli)

Basionym: *Colletotrichum acutatum var. fioriniae* (Marcelino & Gouli)

Originally the name to identify *C.fioriniae* was *Gnomoniopsis rubicola* (Stoneman, 1898), belonging to a group of five species, including *G. cingulata*, on which the genus *Glomerella* was based (Schrenk & Spaulding, 1903) and it was discovered for the first time from *Rubus strigosus* leaves in West Virginia (Damm *et al.,* 2012).

Later, strains now identified as *C.fioriniae* wasere called *C. acutatum;* they were implicated in fruit rot of cranberry and blueberry throughout the northern USA and in British Columbia (MacKenzie *et al.,* 2009; Polashock *et al.,* 2009).

The name *C.fioriniae* which derives from *C.acutatum* var.*fioriniae* (Marcelino *et al.,* 2008; Damm *et al.,* 2012), was assigned for a series of strains isolated from an epizootic infection of the exotic scale insect *Fiorinia externa* in the New England region (Damm *et al.,* 2012). *C. fioriniae* is characterised by a large number of strains and has been isolated from a wide variety of host plants highlighting heterogeneity within the species.

That species is an endophyte (Marcelino *et al.,* 2009) both in the host plant of the insect (sap-suckers) and in a phylogenetically diverse set of associated plants.

There are a lot of host plants of *C. fioriniae*, such as almond, apple, avocado, mango, nectarine (Guerber *et al.,* 2003) on which it causes anthracnose (Damm *et al.,* 2012).

C.fioriniae: morphological and cultural characteristics

As reported by Damm *et al.,* 2012, vegetative hyphae have 1.5–7.5 µm of diameter, they are hyaline to pale brown, smooth-walled, septate and branched. The *conidiomata* are formed directly by hyphae while the *chlamydospores* and the *setae* are not observed.

Conidiophores are hyaline to pale brown, smooth-walled and septate, branched up to 35 µm long. *Conidiogenous* cells are hyaline to pale brown, smooth-walled, cylindrical to ampulliform, sometimes lacking a basal septum and they are continuous with the conidiophore and sometimes covert with a mucous coating.

Conidia are hyaline, smooth-walled, aseptate, straight, fusiform to cylindrical with both ends

acute, (10–)13.5–16.5(–19.5) × 4–5(–5.5) µm, mean ± SD = 15.0 ± 1.6 × 4.5 ± 0.3 µm, L/W ratio = 3.3 µm.

Appressoria are solitary or in loose groups, pale to medium brown, smooth-walled, ellipsoidal, clavate to irregular outline, entire edge or undulate, (4.5–)7–11.5(–15.5) × (4–)4.5–7(–10.5) µm, mean ± SD = 9.2 ± 2.2 × 5.6 ± 1.2 µm, L/W ratio = 1.6.

When *C.fiorinae* colonies grow on filter papers (SNA), they appear with white to pale olivaceous grey aerial mycelium and partly with salmon to orange acervuli, they reverse filter paper with pale olivaceous grey to olivaceous grey patches and spots, and they have a growth rate of about 22.5–23 mm in 7 days (32.5–34 mm in 10 days). When they grow on OA (Oeatmeat Agar), colonies are flat with entire margin; they show a surface saffron with olivaceous spots (mottled) covered with salmon acervuli, aerial mycelium lacking. Their growth rate is about 22–22.5 mm in 7 days (34–35 mm in 10 days).

Objectives and approach of the project

Objectives - The overall goal of this work is to investigate the necrosis found in some Italian stands of *Castanea sativa* Mill., both in natural and artificial infections of *Dryocosmus kurophilus* and identify the causative agent associated with galls and rotten nuts of chestnuts in northern Italy.
To achieve these goals it is therefore necessary : a) monitoring the symptoms; b) to collect isolates of the pathogens from the lesions on *Castanea sativa*; c) to characterize the species using both morphological and molecular techniques; d) to carry out in vitro experiments.

Approach of the project - In Chapter 1 the necrotic galls produced by *Dryocosmus kuriphilus* were investigated in order to identify the causative agent associated. *Colletotrichum acutatum* (J.H. Simmons) was detected.
In Chapter 2 the pink-coloured tissues observed in rotting chestnut nuts were studied. Also in the affected pink-coloured fruits *Colletotrichum acutatum* (J.H. Simmons) was detected. Koch's postulates were applied and confirmed the ability of the pathogen to colonize nuts.
In Chapter 3 a deeper analysis of the *Colletotrichum* isolates was performed.
In this context the genetic diversity of the *Colletotrichum* isolates from *C. sativa* using DNA barcoding was investigated. Analyses were carried out on multilocus sequences of both ribosomal and non-ribosomal genes.

I-Chapter

Colletotrichum acutatum associated with *Dryocosmus kuriphilus* galls on *Castanea sativa*

Abstract

The occurrence of *Colletotrichum acutatum* J. H. Simmonds in necrotized galls of *Dryocosmus kuriphilus* Yasumatsu in chestnut (*Castanea sativa* Miller) stands is reported for the first time in Italy. Morphological and bio-molecular analyses confirmed the isolation of the fungus. No damage by the fungus has been observed.

Introduction

Dryocosmus kuriphilus Yasumatsu, the Asian chestnut gall wasp, has caused great concern for *Castanea sativa* Mill. orchards and stands in Italy since its arrival in 2002.

The larvae of *Dryocosmus kuriphilus* induce the formation of galls on the tree and cause reduction in plants vigor and chestnut production. *D. kuriphilus*, in fact, disrupts twig growth and reduces fruiting; severe infestations may result in the decline and death of chestnut trees (Dixon *et al.,* 1986).

The biological control, obtained with the introduction of the specific parasite *Torymus sinensis* Kamijo, began in Italy in 2005. The increase of parasitoid populations has been exponential, surpassing 90% in 5-7 years after the release (Quacchia *et al.,*2008). This led to a steady decrease of Asian chestnut gall wasp, reducing the degree of infestation and to a gradual improvement in the containment of the insect spread.

The aim of the Plant Protection Service is to supervise the release of the antagonist insect and to constantly monitor the progress by verifying the effectiveness of biological control. Monitoring the presence of the galls and *Torymus sinensis*, it was observed that also fungi may act as natural parasites of gall wasp colonizing the larva of the insect. Some studies, report the presence of *Gnomoniopsis* sp. associated with chestnut gall wasp gall necrosis and gall wasp mortality was reported in the area of Monti Cimini in Central Italy (Magro *et al.,* 2010). Necrotic galls containing dead insects and invaded by unidentified fungi were observed in the United States (Cooper and Rieske, 2007; 2010).

The interest in natural constraints factors against this invasive pest is high and focused also on fungi able to both colonize galls and parasitize this insect pest (Addario *et al.,* 2011).

Materials and methods

Collection of gall samples

In 2013 and 2014, surveys were carried out in chestnut stands located in different province of Lombardy and Trentino. We collected necrotic galls, the 60% of which contained dead *Dryocosmus kuriphilus* individuals.

The galls analyzed presented necrosis of the tissues, darkening and principle of deterioration. (Photo 9A-B; Photo 10). Before isolation the material was sterilized with 1% hypochlorite and portions of material were taken through a scalpel, for subsequent analysis.

Isolation and Morphology analysis

Potato dextrose agar with biotin (1 mg/l) and methionine (100 mg/l) (PDAmb) was used for isolations in Petri dishes from infected gall tissues and from bodies of some mycelium-enveloped dead cynipids.

Portion of infected gall tissues and mycelium-enveloped dead cynipids were transferred on Potato dextrose agar with biotin (1 mg/l) and methionine (100 mg/l) (PDAmb) in Petri dishes.

Isolations were incubated for 7-8 days (25 °C) in the dark. Six strains were selected in relation to their ability to sporulate on the surface of galls and were subcultured on PDAmb. Single-spores cultures were obtained by plating dilution series on water-agar and transferring germinated conidia on PDAmb after 24 hours of incubation.

Molecular analysis

The mycelium was collected in sterile 1,5 tube, gently scratching the surface of the colony with a sterile scalpel and place in the freezer -20 up to complete freezing.

Nucleic acids were extracted using a commercial kit (NucleoSpin® Plant II,MN GmbH & Co. KG), about 100mg of mycelium was placed in microfuge tubes containing sterile sand and 500 uL of extraction buffer containing 100mM Tris, pH8.0, 10mM EDTA, 2% SDS, 100ug/mL Proteinase K and 1% B-mercaptoethanol; the DNA was precipitate to isopropanol and resuspend in 100uL TE buffer. After, the DNA was quantified by *NanoDrop* 2000, UV-Vis spectrophotometers used to assess purity of DNA.

The DNA was amplified by PCR using primers ITS1 and ITS4 (White *et al.,*1990) (Fiure 2; Table 3).

The 50-µl PCR mixture contained 10 µl of DNA template, 6 µl of 25 mM MgCl2, 5 µl of PCR buffer without MgCl2; 200 µM each deoxynucleoside triphosphate, 25 pmol of each primer, and 1 U of *Taq* DNA polymerase (GoTaq G2 Flexi DNA Polymerase; Promega) (Table 1A-.1B) Reactions involved cycle at 95°C for 5 min, followed by 35 cycles with a denaturation step at 95°C for 30 s, annealing step at 55°C for 1 min, and extension step at 72°C for 1 min, followed by 1 cycle at 72°C for 6 mins (Consuelo *et al.,* 2001).

Two negative controls were included in the amplification, in particular a reagent control (sterile water) and a sample extraction control. The sample extraction control consisted of sterile MilliQ water subjected to the same extraction procedures as the specimens.

10 µl of each amplified product were electrophoretically separated in a 1,2% agarose gel in 1× Tris-borate-EDTA buffer (TBE buffer) and visualized using ethidium bromide under UV illumination. Molecular weight ladders were included (Gene Ruler 100-bp and 1KB DNA Ladder Plus [MBI Fermentas, Vilnius, Lithuania).

PCR products were purified to remove excess primer using NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL GmbH & Co. KG) and then directly sequenced with ABI 3730xl DNA Analyzer systems (96 capillary instrument) (GATC Biotech AG -Germany).

The obtained sequences were analyzed and corrected with BioEdit Sequence Alignment Editor and NCBI GenBank was used for the identification.

Inoculation test

Infection trials were carried out with 1 tested isolate out of 25 not necrotic Asian chestnut gall wasp galls collected in spring 2014: several conidia suspensions at different concentrations (5 x 103, 5 x 104 and 5 x 105 conidia/ml) were sprayed on the galls that were then kept in humid chamber for 7 to 10 days at 23-25°C; as negative control, 10 additional galls were treated with sterile water.

A B

Photo 9A-B (F.Gaffuri): Symptoms on galls analyzed. The photo show necrosis of the tissues, darkening and principle of deterioration.

Photo 10: (Gaffuri *et al.,*2015). Necrotized gall of *Dryocosmus kuriphilus*.

Figure 2: ITS gene region lies between the small subunit (SSU) and the large subunit (LSU) ribosomal RNA (rRNA) genes and contains two noncoding spacer regions (ITS-A and ITS-B) separated by the 5.8S rRNA gene.

Primer	Sequence	Target gene	Reference
ITS1	5'-TCC GTA GGT GAA CCT GCG G-3'	Fungal ITS	White *et al.,* 1990
ITS4	5'-TCC TCC GCT TAT TGA TAT GC-3'	Fungal ITS	White *et al.,* 1990

Table 3 : Primer sets used for ITS gene PCR amplification and sequencing designed by White *et al.,* (1990). In fungi, the ITS region is typically 650–900 bp in size.

Results

Colony morphology and isolation results

Cultures grown on PDA plates incubated at 25°C produced mycelia that are initially white turning into gray and pale orange or pink (Photo 11).

The conidia obtained from acervuli in the cultures are hyaline, aseptate, fusiform, ranging from (8.9–) 11–14.7 (–15.2) × 3.0–4.5(–5) µm, mean ± SD = 12.3 ± 1.4 × 3.8 ± 0.5 µm, L/W ratio = 3.2 µm.

The colony diameters (mm) were recorded every day for 1 week to determine the growth rates of each isolate (Photo4), the lengths and widths of at least 50 conidia were measured, and the colony colour was recorded. The size, shape and colour of the conidial masses and other key characteristics of each structure were also scored.

Photomicrographs were taken with a digital camera. The morphological characteristics that were observed of the fungus were similar to those described for the *Colletotrichum acutatum* J. H. Simmonds species complex (Damm *et al.,* 2012).

Inoculation test

Ten days after the inoculation, initial necrosis were observed on all of the inoculated galls, with evident mycelia and an abundant production of conidia in a gelatinous matrix in three cases (Photo 12). The re-isolation on PDA produced colonies and conidia of *C.acutatum*, as well as the conidia obtained from galls showed shape and dimensions similar to those of the original cultures. No symptoms and fungal growth were observed on the untreated galls.

Molecular analysis

Amplifications were successfully carried out with the ITS1⁄ITS4 primers and fragments of approximately 580 bp were obtained.

Sequencing results confirmed that 35 isolates shown nucleotide sequence identities of 100% for *C.acutatum* isolate AB233348.1 (from GenBank database) and other genus and species, in particular: *A.alternata*, *F.oxysporum, F.accuminatum, Cryphonectria parasitica, Botrytis, Trichoderma viride, Gnomoniopsis* and *Glomerella acutata.*

All DNA sequences were deposited in GenBank with accession numbers from KP064131 to KP064137.

Photo 12 (Gaffuri *et al.,*2015). Infection test: mycelia and orange spore masses of *Colletotrichum acutatum* on treated galls.

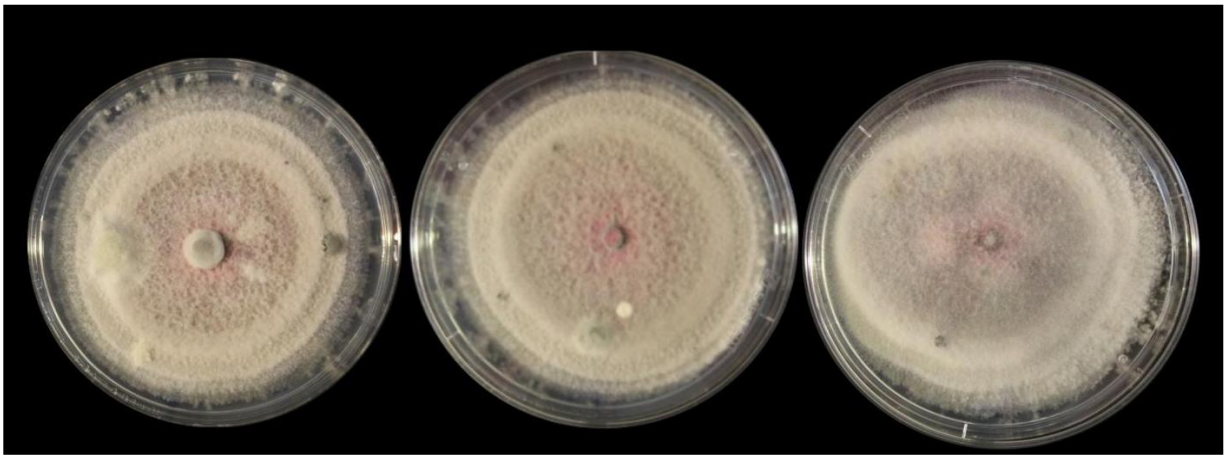

Photo 11 (Gaffuri F.): Cultures of *Colletotrichum* grown on PDA plates incubated at 25°C produced mycelia that were first white and then gray and pale orange or pink.

Discussion

C. acutatum can cause a wide range of pre- and postharvest anthracnose diseases on economically important crops. Although the fungus has been reported to infect vegetative tissues of woody,herbaceous crops, conifers ornamental and forage plants (Strandberg 2001), it is an endophyte and it is capable of colonizing insects too (MacKenzie *et al.,* 2009; Marcelino *et al.,* 2008).

So far, no pathogenic symptoms were observed on chestnut leaves and stems. However, the gall tissues, which are very rich in starch (Ugolini *et al.,* 2014), were proved to be easily colonized by *C. acutatum*, enhancing the chances for this pathogen to survive and spread also on chestnut trees. This might increase the potential spreading of this fungus, because of the huge and continuous chestnut trange in Italy and the abundance of galls still present on trees.

The first discovery of *C. acutatum* was observed in Lombardia and Trentino, and from monitoring data, this fungus was found to be spread in all chestnut-growing areas monitored on necrotic galls. *C. acutatum* has also been found on cultivated and weeds plants near chestnut trees and was characterized by a typical symptomatology, that is anthracnosis with subsequent and progressive necrosis of green tissues. However, these symptoms were not observed on chestnut. Similar study, carried out in Sondrio province, also showed the presence of *Trichoderma viride* on *D. kuriphilus* necrosis, while *C. acutatum* was found in the same area but on healthy galls.

This observation was noteworthy as *Trichoderma* spp. are among the most studied fungal BCAs and commercially marketed as biopesticides, biofertilizers and soil amendments (Harman, 2000; Harman *et al.,* 2004; Lorito *et al.,* 2004).

This has led to arrange tests on the interaction between *C. acutatum* and *T. viride*. Results of the experiment, however, showed no antagonistic activity between *T. viride* and *C. acutatum*. The same result was obtained using other *Trichoderma* spp. isolates such as *T. asperellum*, *T. gamsii* and *T. harzianum* against *C. acutatum*.

Since this study represents the first reporting of *C. acutatum* in chestnut, other molecular analysis on barcode genes in addition to ITS are necessary to confirm the identification of *Colletotrichum.* Moreover, the molecular analysis showed the presence of the holotype *Glomerella acutata*, an interspecific hybrid between *C. acutatum* and *C. fioriniae* (Vaillancourt *et al.,* 1992).

Note & Acknowledgements

The chapter 1 describes in detail the major themes that emerged from my PhD research published on Forest Pathology and reported below For. Path. 45 (2015) 169–171 doi: 10.1111/efp.12178. title: *Colletotrichum acutatum* associated with *Dryocosmus kuriphilus* galls on *Castanea sativa.*

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II-Chapter

"Pink rot": infection of *Castanea sativa* fruits by *Colletotrichum acutatum*

Abstract

Pink-coloured tissues were observed in rotting chestnut nuts collected from soil in different orchards in Italy (Emilia Romagna, Tuscany and Trentino). Morphological and molecular analysis confirmed the presence of *Colletotrichum acutatum* (J.H. Simmons) in affected fruits, and inoculation tests corroborated the ability of the pathogen to colonize nuts.

Introduction

Chestnut cultivation in Italy and in Europe is of particular importance because it is necessary to the economy of many mountain communities as elements of the landscape, sources of timber and, for the production of nuts (Maresi *et al.,* 2013). The quality of chestnuts is determined by size, shape, color, flavor and texture, features which are very important for both fresh consumption and processing (Korel *et al.,* 2008) and which are essential to ensuring an advantageous income for growers.

Unfortunately, chestnut fruit production and quality have strongly declined during the last years by the influence of the cumulative effects of either the spread of the Asian Chestnut Gall Wasp (ACGW) (*Dryocosmus kuriphilus* Yasumatsu) or the irregular weather (Battisti *et al.,* 2014). Environmental factors can also influence the quality of fruits: low soil water availability and high temperatures are destabilizing factors for normal chestnut growth (Maresi *et al.,* 2013).

The impact from *D. kuriphilus* in Europe on yield reduction in *Castanea* was estimated as moderate in the last available review (EFSA Panel on Plant Health, 2010) moreover, these data are confirmed by the study of Battisti *et al.,* 2014 where it is observed evidence of a relationship between gall density and yield loss.

It should be remembered that there are other pathogens and parasites commonly found in our areas that can contribute to the decrease or loss of production.

There are indeed considerable problem for chestnut cultivation may arise from the fact that nuts, contain sugars (monosaccharides and disaccharides) sucrose, glucose, fructose and raffinose, as well as starch (Bernardez *et al.,* 2004) and they are highly attractive to insects and fungi, which can cause severe damage.

They are typical attacks by insect larvae, as the are well known to be detrimental to the harvesting of healthy chestnut fruits e.g. *Cydia splendana* Hb., *Cydia fagiglandana* Zell. and *Curculio elephans*

Gyll. (Pedrazzoli *et al.,* 2012) and also, several fungi are capable of colonizing nuts. In particularly we can found: *Phoma endogena* and *Phomopsis endogena* (described as agents of "brown rot"), *Sclerotinia pseudotuberosa* Rehm (black rot), *Penicillium* spp. and *Penicillium crustaceum* L. Fr. (produce greenish moulds).

Each of the organisms mentioned above exhibits a characteristic symptom that identifies the his presence in/on C*astanea sativa* but the recent appearance of the invasive Asian chestnut gall wasp in chestnut stands showed an unusual level of fruit damage was reported in Piedmont (Maresi *et al.,* 2013), more or less in correspondence with the wasp invasion, (Gentile *et al.,* 2010) and subsequently in other chestnut-producing areas of Italy. The damage in Piedmont was initially attributed to in *Gnomonia pascoe* (Gentile *et al.,* 2013) and recently related to *Gnomoniopsis castanea*, a proposed new species (Visentin *et al.,*2012). Furthermore, *Gnomoniopsis* sp. has been found to be associated with necrosis of *D. kuriphilus* galls, symptoms showed lesions on leaves were irregular and variable in size, lemon green to amber in colour with green margins. Initially, galls were olive green then became dark brown. Inside the galls larvae of chestnut gall wasp were dead (Magro *et al.,* 2010).

In this context, chestnut orchards in various parts of Italy have been investigated to evaluate the role of insect and fungal damage on fruit. During this survey, single or few nuts showed a still undescribed symptom: a clear and sometimes intense pink coloration of the endosperm. This was generally the main cause of nut decay although sometimes associated with brown rot symptoms (Maresi *et al.,* 2013). 'Pink rot' has never been reported previously as a chestnut rot symptom; therefore, an investigation was carried out to clarify the causal agent of this new symptom (Gaffuri *et al.,* 2016).

During this study, was identified a *Colletotrichum acutatum*, this organism has been already reported as an endophyte on chestnut branches (Bissegger & Sieber, 1994), and recently demonstrated the ability of the fungus to colonize galls and adults of *Dryocosmus kuriphilus* Yasumatsu (Gaffuri *et al.,* 2015). The discovering of this almost worldwide pathogen associated to a new symptomatology on chestnuts confirms a possible risk related to its presence, because the endophytic isolates showed the same pathogenicity of those obtained from infected nuts.

Materials and methods

Collected material

During the monitoring, have been identified particular symptoms on nuts. We collected these fruits in different orchards in the province of Tuscany, Casentino (Ar); Tuscany, Mugello (Fi); Emilia Romagna, Montese (Mo); Castione (Tn); Drena (Tn); Tenno (Tn); Tuscany (2 nuts) (Photo 13)

Each fruit was dissected and analyzed by stereomicroscope and after having peeled the nuts, tissue from pink decaying endosperm was surface-sterilized for 1 min in 70% ethanol, 5 min in 1.25% sodium hypochlorite and 30 s in 70% ethanol.

The selected material were rinsed twice in sterile distilled water and blotted dry on sterile filter paper. Fragments of tissue were then plated on Potato Dextrose Agar (Difco, USA) and incubated in the dark at 25 ± 1°C for 1 week.

The colonies were purified in subcultures in the same conditions and the plates were analyzed by stereo-microscopic for the identification of morphological characteristic structures.

In addition to the collected material, Healthy and surface-sterilized nuts were artificially infected with four different isolates of *C. acutatum*, two obtained from affected nuts and two previously isolated from healthy looking chestnut branches collected in a different orchard where no symptoms were observed.

An artificial injury was made in the pericarp near the hilum, into which a fragment of mycelium was placed and covered with masking tape; the inoculated nuts were then stored at 25°C in the dark. Twelve replicates were carried out for each isolate, and 12 wounded but untreated nuts served as controls.

Molecular analysis

Nucleic acids were extracted from 10-day-old cultures: the mycelium was collected in sterile 1,5 tube, gently scratching the surface of the colony with a sterile scalpel and place in the freezer -20 up to complete freezing.

Nucleic acids were extracted using a commercial kit (NucleoSpin® Plant II,MN GmbH & Co. KG), about 100mg of mycelium was placed in microfuge tubes containing sterile sand and 500 uL of extraction buffer containing 100mM Tris, pH8.0, 10mM EDTA, 2% SDS, 100ug/mL Proteinase K and 1% B-mercaptoethanol; the DNA was precipitate to isopropanol and resuspend in 100uL TE buffer.

After, the DNA was quantified by *NanoDrop* 2000, UV-Vis spectrophotometers used to assess purity of DNA.

The DNA was amplified by PCR using primers ITS1 and ITS4 (White *et al.,*1990).

The 50-µl PCR mixture contained 10 µl of DNA template, 6 µl of 25 mM MgCl2, 5 µl of PCR buffer without MgCl2; 200 µM each deoxynucleoside triphosphate, 25 pmol of each primer, and 1 U of *Taq* DNA polymerase (GoTaq G2 Flexi DNA Polymerase; Promega). Reactions involved 1 cycle at 95°C for 5 min, followed by 35 cycles with a denaturation step at 95°C for 30 s, annealing step at 55°C for 1 min, and extension step at 72°C for 1 min, followed by 1 cycle at 72°C for 6 mins (Consuelo *et al.,* 2001).

Two negative controls were included in the amplification, in particular a reagent control (sterile water) and a sample extraction control. The sample extraction control consisted of sterile MilliQ water subjected to the same extraction procedures as the specimens.

10 µl of each amplified product were electrophoretically separated in a 1,2% agarose gel in 1× Tris-borate-EDTA buffer (TBE buffer) and visualized using ethidium bromide under UV illumination. Molecular weight ladders were included (Gene Ruler 100-bp and 1KB DNA Ladder Plus [MBI Fermentas, Vilnius, Lithuania).

PCR products were purified with illustra ExoProStar 1-Step (GE Healthcare, UK), PCR products were sequenced with the Big Dye terminator v3.1. cycle sequencing kit (Applied Biosystems,Foster City, California) on an Applied Biosystems 3130xl Genetic Analyzer.

BLASTN comparison of the sequences of the amplicons was performed using the NCBI database to confirm the identity of isolates.

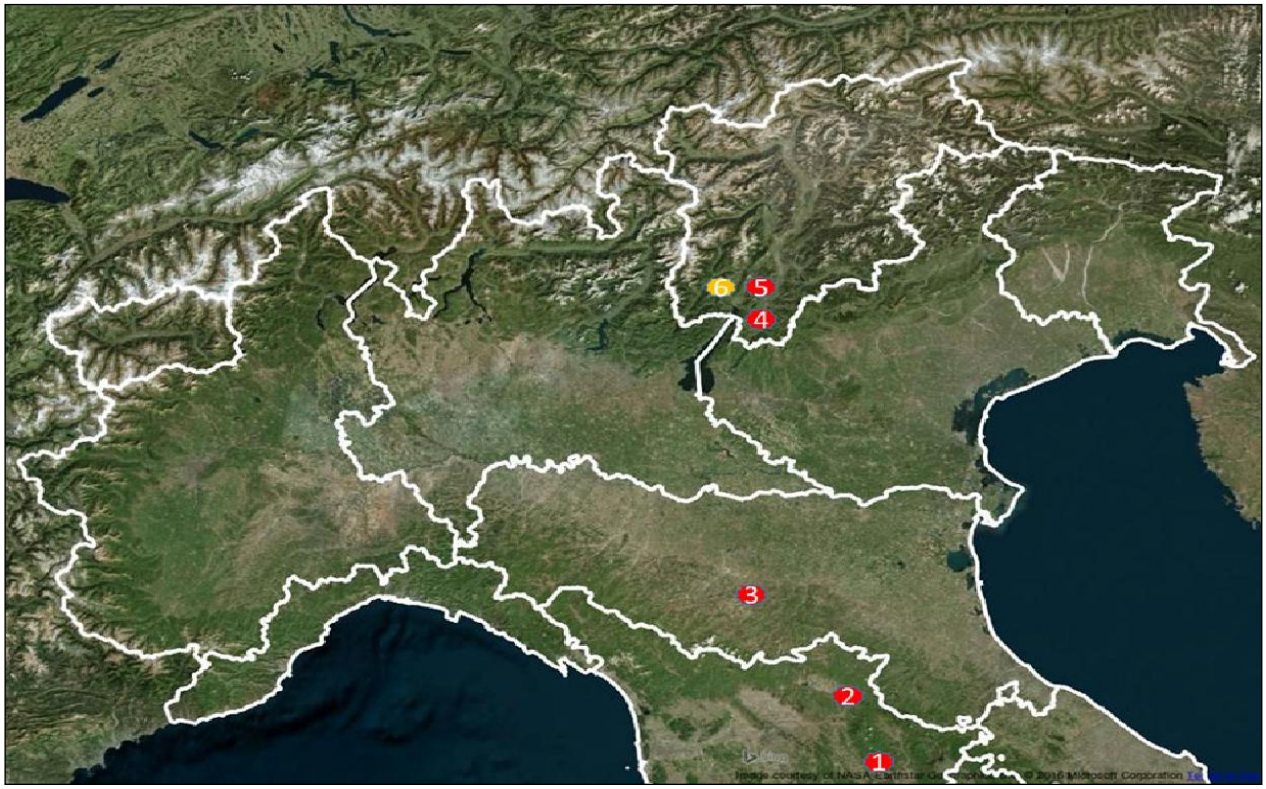

Photo 13 (Gaffuri *et al.,*2016): Maps of the orchards where infected nuts were collected: 1 – Tuscany,Casentino (Ar), 2 – Tuscany, Mugello (Fi); 3 – Emilia Romagna, Montese (Mo), 4 –Castione (Tn), 5 – Drena (Tn), 6 – Tenno (Tn) (healthy branches).

Results

Colony morphology and isolation results

The isolates were cultured on potato dextrose agar PDA in darkness at 25°C and the vegetative and reproductive structures were described after 10 days of incubation.

From 75 up to 90% positive isolations of morphological similar cultures were obtained from all assayed nuts, with no difference between the areas of collection.

Mycelia first appear white, then grey and pale orange or pink; conidia were taken from actively growing colonies and suspended in sterile water. Length and width were measured for 100 conidia. They showed up: hyaline, aseptate, fusiform, and ranging from (8.7–) 11–14.6 (−15.2) × 3.0–4.5 (−5) µm, mean ± SD = 12.2 ± 1.3× 3.8 ± 0.5 µm, L/W ratio = 3.2. Morphological characteristics of conidia and colony were compared with all isolates.

The morphological characteristics that were observed of the fungus were similar to those described for the *Colletotrichum acutatum* species complex (Damm *et al.,*.2012).

Results of inoculation test

Thirty days after inoculation, the characteristic pink coloration was observed in 65–70% of the inoculated nuts (Photo 14), without significant differences between the tested isolates, while the control nuts contained no rot tissues. The formed mycelium, was taken with a sterile scalpel and placed in new plates containing PDA agar and produced colonies of *Colletotrichum acutatum* sensu lato morphological similar to those of the original cultures.

Results of Molecular analysis

BLAST analysis of PCR products of the isolates obtained from pink tissues, carried out with the ITS1/ITS4 primers fragments of approximately 580 bp were obtained.

Amplifications showed 100% of sequence homology with the *C. acutatum* isolate KP064131.1 and KT823767.1 from GenBank database. Two of DNA sequences were deposited in GenBank with accession numbers KX078637 and KX078638. KP064137.

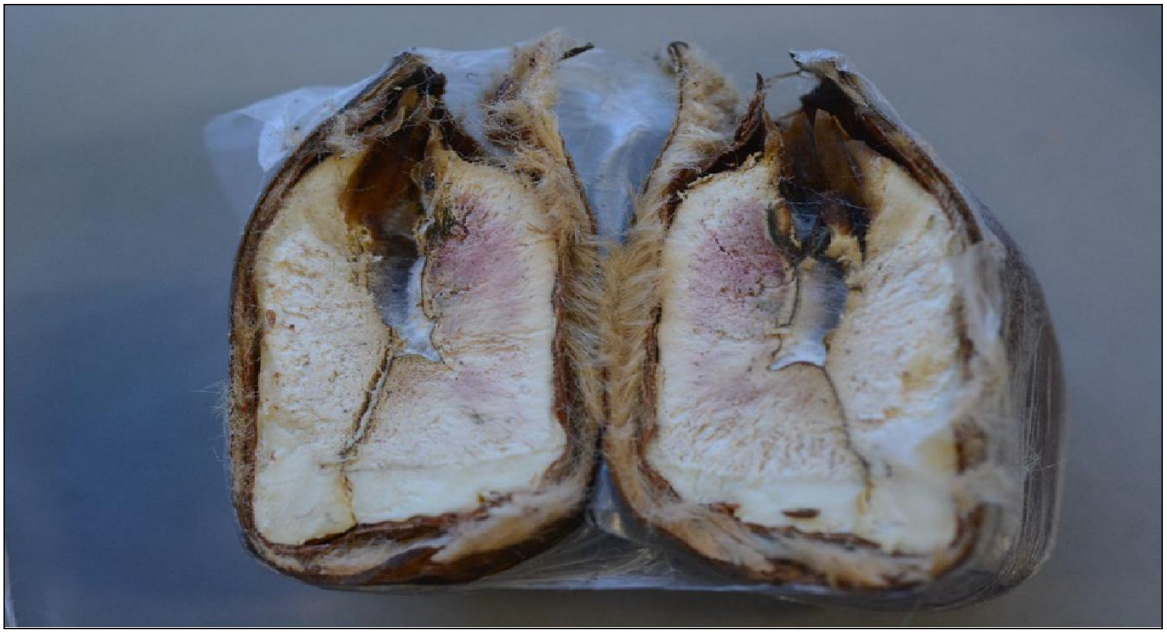

Photo 14 (Gaffuri *et al.,*2016): The characteristic pink coloration observed on nut artificially inoculated with *Colletotrichum acutatum*

Discussion

Many are pathogenic fungi that can cause lesions in the chestnut plant, and the occupation of potential micro-habitats of pathogens by avirulent endophytic might prevent invasion or infection by disease causing fungi (Carroll, 1988; Minter, 1981).

Some of the endophytic fungi were reported to be pathogenic themselves, and thus their potential as natural biocontrol agents may be limited (Bisseger *et al.,*1994). In this point, *Colletotrichum* species are proving to be widespread on a broad range of plant hosts and some are proving to be endophytes as well as pathogens.

Many of the recently described species (Yang *et al.,* 2009) are proving to be widespread on a range of unrelated hosts, some as endophytes, epiphytes and pathogens, or as weak or opportunistic pathogens.

C. acutatum has been already reported as an endophyte on chestnut branches (Bissegger & Sieber, 1994), and recently Gaffuri *et al.,* (2015) demonstrated the ability of the fungus to colonize galls and adults of *Dryocosmus kuriphilus* Yasumatsu.

Generally *Colletotrichum* cause in some plants symptoms that consist of dark, elongated lesions also in field warm and wet conditions are present, such lesions will contain orange masses of the pathogen's spores. For below-ground parts, symptoms consist of decayed, darkened roots, discoloration of the internal crown tissue, and wilting and collapsing plants (Bolda *et al.,* 2016).

However, these symptoms have never been identified on castanea and therefore there is still no correlation between the presence of C*olletotrichum* and symptom.

The finding of this almost worldwide pathogen (Damm *et al.,*2012) associated to a new symptomatology, characteristic pink coloration of nuts, confirms a possible risk related to its presence, because the endophytic isolates showed the same pathogenicity of those obtained from infected nuts.

Luckily, a very low level of infection has been recorded till now in all investigated chestnut stands (seven affected nuts of 15,000 damaged ones examined in 2015 in Trentino) (Gaffuri *et al.,* 2016).

The recent findings on this study, suggested some concern about the role of *C. acutatum* in the chestnut ecosystem, even if evidence of damage on stem and leaves has yet to be observed and few data have been till now obtained about the real consistency on the presence of the fungus on chestnut trees. Future investigations are needed to confirm whether ‘pink rot’ could be

considered a real threat for chestnut production and whether there is a possible role of *C. acutatum* in influencing the ecology and productivity of chestnut stands.

Note & Acknowledgements

The chapter 2 describes in detail the major themes that emerged from my PhD research published on Forest Pathology and reported below (For. Path.(2016) DOI: 10.1111/efp.12307) title: "Pink rot": infection of *Castanea sativa* fruits by *Colletotrichum acutatum*.

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III-Chapter

Molecular identification and diversity assessment of *Colletotrichum spp* infecting *Castanea sativa* in northern Italy

Abstract

Colletotrichum is a common genus of fungi infecting a wide array of crops and non-cultivated plant species and it is known as an important pathogen causing anthracnose. Recently in Italy, the fungus was recovered on chestnut either on Asian gall wasp galls or on nuts, confirming a previous record reporting its endophytic behaviour.

Because of its complexity, not already completely clarified, a survey was carried out within the genus to clarify which species are responsible for chestnut infection. *Colletotricum sp* was detected in 19 out of 40 investigated orchards from healthy shoots bark and from galls, both necrotic and healthy. A multi-barcoding approach on both ribosomal (internal transcribed spacer, ITS) and protein-coding (β-tubulin, TUB, and calmodulin, CAL) sequences was adopted for species identification. Resolution till the species level within the *Colletotrichum* complex was only allowed by the combined analyses on multigene sequences: the combination of ITS, TUB and CAL gene analysis identify isolates as *Colletotrichum fioriniae* in all the sites.

Considering the potential pathogenic role of this fungus, its presence on healthy chestnut trees opens interesting views on its ecologiCAL role and impact both on chestnut ecosystem and on other host.

Introduction

Castanea sativa Mill. (genus *Castanea,* family *Fagaceae)* is widespread in Europe, where it has both an economical and environmental importance. Its distribution ranges from Southern Europe and North Africa to North-Western Europe and eastward to Western Asia (Conedera *et al.*, 2016), with an altitudinal range between 200 and 1800 m, depending on the latitude and site aspect (Conedera *et al.*, 2016). The importance of *C. sativa* is ascribable to fruit and timber production, and to the strong cultural legacy due to its fundamental role in modeling economy and landscape, for the Mediterranean mountain people, during several centuries (Pezzi *et al.,* 2017). Still now, chestnut orchards play a key role in the economy of several areas, while landscape values are appreciated and valorized by tourism. Moreover, the ecological value of old growth forests, and generally of chestnut habitat, has been recognized by the European Union (Habitat Directive 92/43/EEC).

Sweet chestnut is one of the best examples of forest trees really threatened by invasive pathogens and insect pests, that represent an increasing problem for forests and other ecosystems (Santini *et al.,* 2013). First *Phytophthora cambivora* [Petri] Buism.) in the XIXth century, later *Cryphonectria parasitica* [Murr.] Barr), in the second half of the XXth century, caused great concern for the survival of stands and orchards. Last, *Dryocosmus kuriphilus* Yasumatsu, the Asian chestnut gall wasp, spread and quickly colonized all the chestnut range causing important losses in different countries among which Italy, where it was first reported in 2002 (Brussino *et al.,* 2002). A variety of concomitant events among which environmental factors for ink disease (Turchetti and Maresi, 2008), hypovirulence for chestnut blight (Turchetti *et al.,* 2008) and biological control by parasitotid (Quacchia *et al.,* 2008) for Asian wasp have allowed the chestnut to survive this massive invasion.

In 2014 the occurrence of *Colletotrichum acutatum* J. H. Simmonds in necrotized galls of *D. kuriphilus* in chestnut stands was recorded for the first time in Italy (Gaffuri *et al.*, 2015). Previously Bissegger and Sieber (1994) reported the isolation of *C. acutatum* from coppice shoots chestnut bark in canton Ticino (Swiss). Moreover pink-coloured tissues were observed in rotting chestnut nuts collected from soil in different orchards in Italy (Emilia Romagna, Tuscany and Trentino) (Gaffuri *et al.,* 2016). Morphological and molecular analysis confirmed the presence of *Colletotrichum acutatum* (J.H. Simmons) in affected fruits, and inoculation tests corroborated the

ability of the pathogen to colonize nuts on *Castanea sativa.*

Colletotrichum spp. have a wide host range and cause anthracnose on numerous host plants worldwide (Farr and Rossman, 2013). Disease symptoms appear on young and old trees, on leaves, fruits and flowers of several crops of agronomic interest and on several herbaceous plants (Damm *et al.,* 2012). The parasite causes drop of several crops, such as strawberry (Garrido *et al.*, 2009), Acacia (Golzar, 2009), olive (Spooner-Hart *et al.*, 2007) and almond (McKay *et al.*, 2009).

Colletotrichum species acting as endophytes have also been recorded (Dingley and Gilmour 1972, Wang *et al.,* 2008; MacKenzie *et al.*, 2009) and some isolates (*Colletotrichum acutatum fioriniae*) have been detected in mealybugs (Marcelino *et al.,* 2009). Isolates of *C.acutatum* var *fioriniae* were also recovered from *Tsuga canadensis* (L.), a common species in forest in the northeastern United States (Marcelino et al. 2009).

The genus *Colletotrichum* has undergone frequent taxonomic changes in the past decades with the merging and addition of many species (Baroncelli *et al.,* 2017). Species concepts are still in a state of flux, however several major monophyletic clades, or species complexes, are now recognized (Damm *et al.,* 2012).

Identification of *Colletotrichum* spp. has been conventionally performed using classical mycological methods based on morphological characters such as shape and size of conidia, setae, appressoria and sclerotia together with geographical origin and host association patterns. Using this system around 900 species were assigned to the genus (reviewed by Sutton, 1992; Baroncelli *et al.,*2016) However, these criteria alone are often insufficient to differentiate species, due to the variations in morphology and phenotype under different environmental conditions (Than *et al.,* 2008) and to the fact that teleomorphic stages are rarely formed (Hyde *et al.*, 2009).

C. acutatum is in fact now considered as a species complex, with a high genetic and morphological divergence and a wide range of hosts within intra-specific populations (Sutton, 1992). There are many factors that can vary within a complex: host, host tissue infected, and the environment (Peres *et al.,* 2005). Members of the *C. acutatum* species complex cause both pre-harvest and post-harvest diseases (Shi *et al.,* 1996). Within the complex, *Colletotrichum fioriniae* is used as a reference strain for phylogenetic analyses (Baroncelli *et al.,* 2014).

C. fioriniae is associated to different diseases e.g., on leaf and stem blight of *Acacia acuminata*, on fruit rot of *Persea americana*, besides causing postharvest decay on apple fruits (Kou *et al.,* 2014)

and behaving as endophyte in *Mangifera indica* and in 28 other plant species (Shivas *et al*, 2009). Because of the pathological relevance of species belonging to the *C. acutatum* complex*,* it is of outstanding importance to define the isolates involved in chestnut ecosystems and understand their role in the ecology of these woods. In this context we investigated the genetic diversity of the *Colletotrichum* isolates from *C. sativa* using DNA barcoding. Analyses were carried out on multilocus sequences of both ribosomal and non-ribosomal genes.

Materials and methods

Sample collection, isolation and morphological analysis

Samples were collected in 40 different chestnut orchards, randomly chosen in Lombardy and Trentino woods (table 1). Three shoots on three plants containing at least two galls were randomly collected. Samples were placed in plastic bags and stored at 4°C for laboratory essays. Collection of samples was carried out in summer 2015 for Lombardy and in spring 2016 for Trentino.

Isolates were taken from two year old branches and galls (both necrotic and healthy). Samples were surface-sterilised for 1 min in 70% ethanol, 5 min in 1.25% sodium hypochlorite and 30 seconds in 70% ethanol. They were rinsed twice in sterile, distilled water and blotted dry on sterile filter paper (Stanosz *et al.* 2001). Five fragments were cut from various positions on the bark tissue of each sample or from gall and placed on Potato Dextrose Agar (Difco, USA) without antibiotics and incubated in darkness at 25 ± 1° C for 10 days.

All developing fungal colonies were subcultured on PDA without antibiotics in the same conditions. Colonies were then submitted to morphological microscopical investigation, in order to discriminate those belonging to the genus *Colletotrichum;* 29 colonies were selected and submitted to molecular analysis.

Molecular analysis: DNA extraction

Genomic DNA was extracted according to Lee *et al.,* 1988; Wu *et al.,* 2001.

Mycelium (about 500 ug) was placed in micro-centrifuge tubes containing sterile sand and 500 ug extraction buffer (100mM Tris, pH8.0, 10mM EDTA, 2% SDS, 100ug/mL Proteinase K) and DNA was extracted using a commercial kit (NucleoSpin® Plant II, MN GmbH & Co. KG) and following the manufacturer's protocol. DNA concentrations were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Amplification and sequencing

PCR reactions were performed to amplify: (i) the whole ITS region (ITS1-5.8S rRNA-ITS2) using primers ITS1 and ITS4 (White *et al.,* 1990); (ii) a portion of the β-tubulin gene (TUB) with primers T1 and βt-2b (Glass and Donaldson,1995); (iii) a portion of the calmodulin (CAL) gene using primers CAL-737R and CAL-228F (Carbone and Kohn,1999). Primers sequences are listed in Table 4. Reactions were assembled in a total volume of 25 µl using the GoTaq G2 Master Mix (Promega). All the amplications were carried out in a 9700 thermal cycler (Applied Biosystem). The cycling programs are listed hereafter. For ITS: initial denaturation (5 min at 94°C), 40x (45 sec at 94°C, 30 sec at 52°C, 90sec at 72°C), final elongation (6min 72°C). For TUB: 5min 94°C, 40x (45 sec 94°C, 30 sec 52°C, 90 sec 72°C), 6 min 72°C. For CAL: 2min 94°C, 35x (60 sec 94°C, 30 sec 50°C, 90sec72°C), 10min 72°C. PCR products were resolved on 2% agarose gels and analyzed under UV light. Amplicons were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions and sequenced in both directions using an ABI 3730xl DNA Analyzer system (Life Technologies, Foster City,CA).

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Analysis of DNA sequencing data

Electropherograms were visually inspected and checked for quality using the Bioedit software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) (Hall, 1999). A consensus sequence for barcode in all individuals were created by combining the two partially overlapping sequences obtained using each primer. Similarity searches were performed by means of the basic alignment search program within the GenBank database (www.ncbi.nlm.nih.gov/BLAST) and aligned by means of the ClustalW algorithm implemented in MEGA7 (Kumar *et al.,* 2016). Alignments included all the retrieved species belonging *Colletotrichum* genus and reference sequences downloaded from GenBank. These sequences refer *Colletotrichum* parasites isolated from various hosts in different geographical locations, details of reference isolates used in this study are show in table 2. Neighbor joining (NJ) phylogenetic trees were finally obtained by MEGA7 using the Jukes-Cantor model and 1000 bootstrap replicates (Saiton & Nei, 1987)

Table 4

Sequences, references and targets for the primers used for *Colletotrichum spp.* identification.

Primer	Sequence 5'-3'	Direction	Reference	Locus
ITS 1	TCCGTAGGTGAACCTGCGG	Forward	White *et al.,* (1990)	5.8S mrRNA gene with the two flanking internal transcribed spacers (ITS)
ITS4	TCC TCC GCT TAT TGA TAT GC	Reverse	White *et al.,* (1990)	
T1	AAC ATG CGT GAG ATT GTA AGT	Forward	Glass & Donaldson (1995)	Partial β-tubulin gene (TUB)
βt-2b	ACC CTC AGT GTA GTG ACC CTT GGC	Reverse	O'Donnll & Cigelnik (1997)	
CAL-228F	GAG TTC AAG GAG GCC TTC TCC C	Forward	Carbone & Kohn (1999)	Partial calmodulin gene (CAL)
CAL-737R	CAT CTT TCT GGC CAT CAT GG	Reverse	Carbone & Kohn (1999)	

Results

A total of 360 samples from shoots and necrotic or healthy galls were taken in order to determine the presence of fungi. Shoots (as leaves) showed no symptoms but in some cases, the collected galls were necrotic.

After isolation, it was possible to identify the presence of cultures ascribable to *Colletotrichum spp* in nineteen out of the monitored forty *areas* (Table 5). Other fungi, as *Trichoderma* sp, *Fusarium* sp., *Cryphonectria parasitica* and *Gnomognopsis* sp., were instead identified in all the areas.

No *Colletotrichum* coltures were obtained from all the areas belonging to Sondrio and Varese provinces, while for the other sites, 1-2 positive samples were generally detected out of five tested for each area. In four sites of the Trento province (Serci, Drena, Pranzo, Castione) *Colletotrichum* spp. was isolated from necrotic galls and from the shoots back. The presence of *Colletotrichum* on asymptomatic shoots bark and on necrotic galls was then observed in two areas (Le Piane and Valmoresca) out of a total of five monitored in the Bergamo province, and in four areas (Torno, Gravedona, Albese and Livio out of eight in the province of Como. Finally, in the province of Lecco, the fungus was isolated from a apparently healthy gall, while in other six sampling sites (Monte Barro, Oggiono, Ballabio, Introbio, Primaluna and Barzio) *Colletotrichum* cultures were obtained from all the necrotic galls tested.

Table 5.
Monitoring points in Lombardy and Trentino orchards chestnut.

N.Site	Site	Tissue	*Colletotrichum* isolation	Sampling period
1	Como-loc. Torno	Galls, shoots bark	+	2015
2	Como-loc.Gravedona-	Galls, shoots bark	+	2015
3	Como-loc.Albese	Galls, shoots bark	+	2015
4	Como-Loc.Albavilla	Galls, shoots bark	-	2015
5	Como-loc.Livio (1)	Galls, shoots bark	+	2015
	Como-loc.Livio (2)	Galls, shoots bark		2015
6	Como-Loc.Appiano Gentile	Galls, shoots bark	-	2015
7	Como-Loc.Canzo	Galls, shoots bark	-	2015
8	Brescia-Loc.Predone	Galls, shoots bark	+	2015
9	Brescia- Loc.Val Cavallina	Galls, shoots bark	-	2015
10	Brescia-Loc.Valle San Martino	Galls, shoots bark	+	2015
11	Brescia-Loc.Palazzago	Galls, shoots bark	+	2015
12	Bergamo-loc.Clanezzo	Galls, shoots bark	-	2015
13	Bergamo-Loc.Taiozzo	Galls, shoots bark	-	2015
14	Bergamo-loc.Le Piane	Galls, shoots bark	+	2015
15	Bergamo-Valmoresca	Galls, shoots bark	+	2015
16	Bergamo-S.Brigida	Galls, shoots bark	-	2015
17	Sondrio-Loc.Val Gerola	Galls, shoots bark	-	2015
18	Sondrio-Loc.Tirano	Galls, shoots bark	-	2015
19	Sondrio-Loc.Berbenno	Galls, shoots bark	-	2015
20	Sondrio-Loc.Morbegno	Galls, shoots bark	-	2015
21	Sondrio- Loc.Montagna in Valtellina	Galls, shoots bark	-	2015
22	Sondrio-Loc.Colico (1)	Galls, shoots bark	-	2015
	Sondrio-Loc.Colico (2)	Galls, shoots bark	-	2015
23	Varese-Loc. Parco Pineta - Tradate	Galls, shoots bark	-	2015
24	Varese-Loc.Appiano Gentile	Galls, shoots bark	-	2015
25	Varese-Loc.Luino	Galls, shoots bark	-	2015
26	Lecco-Loc.Monte Barro	Galls, shoots bark	+	2015
27	Lecco-Loc.Pusiano	Galls, shoots bark	-	2015
28	Lecco-Loc.Oggiono	Galls, shoots bark	+	2015
29	Lecco-Loc.Ballabio,	Galls, shoots bark	+	2015

30	Lecco-Loc. Introbio,	Galls, shoots bark	+	2015
31	Lecco-Loc. Primaluna	Galls, shoots bark	+	2015
34	Lecco-Loc. Barzio	Galls, shoots bark	+	2015
35	Trento-Castione	Galls, shoots bark	+	2016
36	Trento-Pranzo	Galls, shoots bark	+	2016
37	Trento-Drena	Galls, shoots bark	+	2016
38	Trento-Serci	Galls, shoots bark	+	2016
39	Trento-Nago	Galls, shoots bark	-	2016
40	Trento-Mezzolombardo	Galls, shoots bark	-	2016

Molecular analysis

A multilocus gene sequencing ("multi barcoding") approach was used for the molecular characterization of Colletotrichum isolates. To this aim, both ribosomal and protein-coding barcodes (ITS, TUB, CAL) were sequenced to accurately pinpoint species identity using database matches, e-values, similarity scores and through the distance tree of search results constructed by the GenBank interface. Sequences obtained directly from PCR products were searched in the GenBank database. Sequences were finally aligned and used for phylogenetic analysis using the NJ approach (Saiton *et al.,* 1987) and the Jukes-Cantor model parameter of pairwise distances.

As shown in Tables 3-5 the highest identity scores were generally obtained for two species: Colletothricum acutatum and Colletotrichum fiorinae. In several cases these species resulted equiprobable when using the ribosomal ITS barcode, that on the whole resulted the less discriminating (Table 6). Non-ribosomal barcodes (CAL, TUB), instead, allowed the resolution till the species level (C. fiorinae, Tables 7-8). The situation is presented in detail hereafter.

ITS sequenze

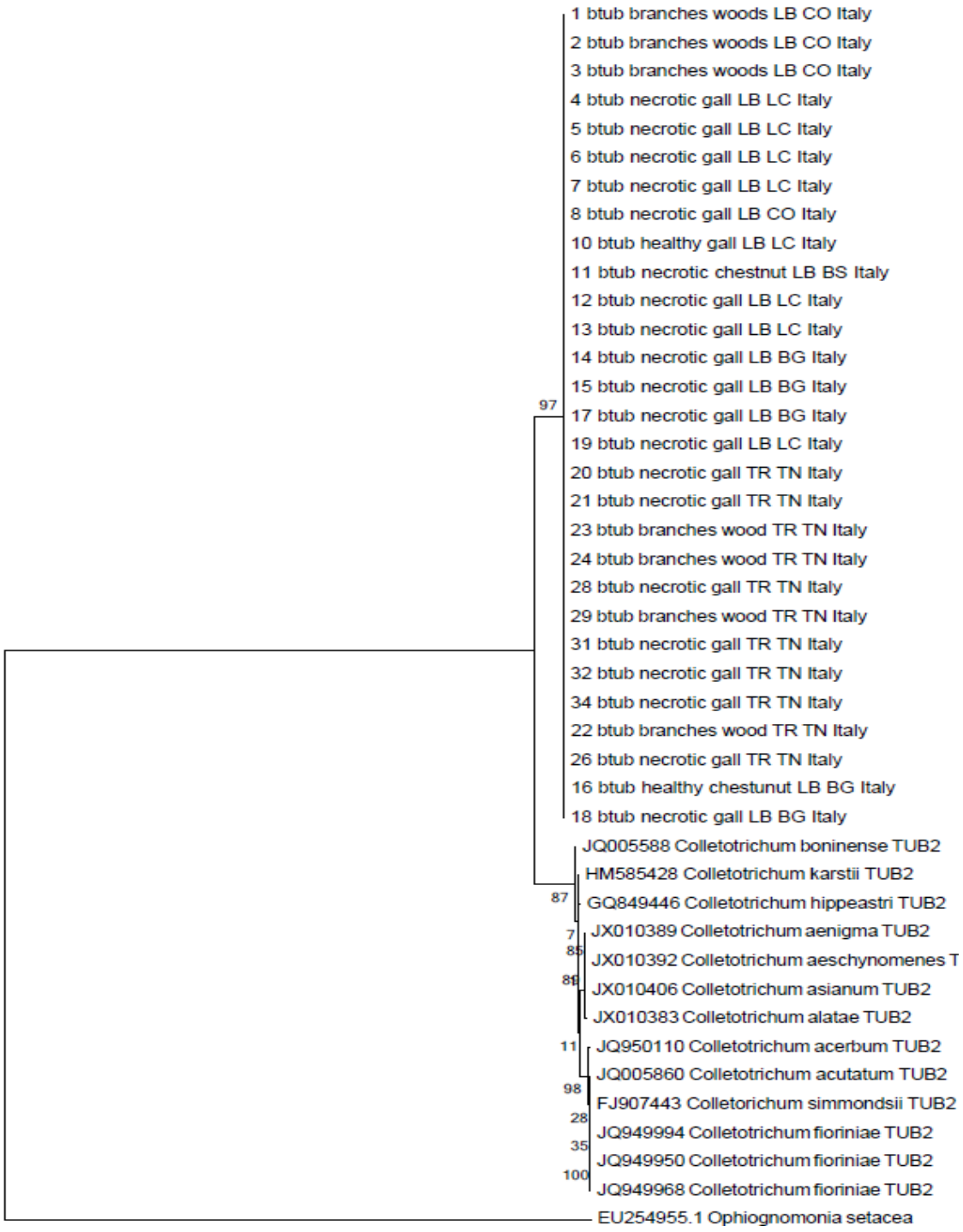
ITS sequences obtained from the collected isolates, when compared with the ones available in the GenBank, yielded as best hits: *C. acutatum*, *C. fioriniae* (often not resolved) and, in a few cases, *Glomerella acutata* (asexual phase of *C. fiorinae). H*omologies were always 99-100%.

In detail: 9 sequences were identified as *C. acutatum* (with KX108948 as best hit), 4 as *C. fioriniae* (best hit: KY271785) and 3 sequences as *G. acutata* (Acc.N. AB233348). The remaining 17 isolates were not resolved, with *C. fioriniae* and *C. acutatum* resulting equiprobable. Details and accession numbers are given in Table 3.

Non ribosomal barcode sequences (CAL and TUB)

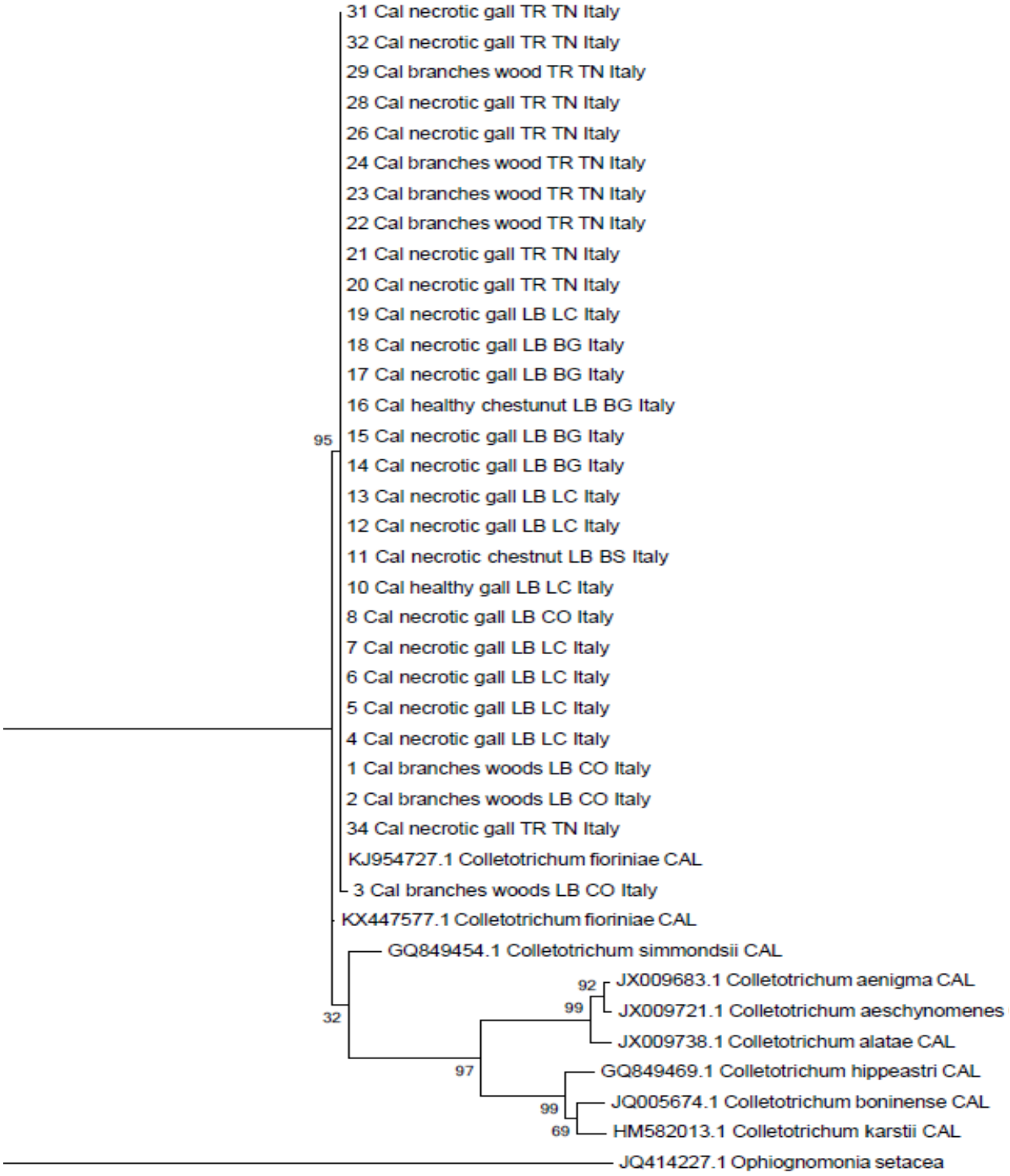
Non-ribosomal loci TUB and CAL were proposed as barcode targets for fungi identification (Damm *et al.,* 2012).

The percentage of identity ranges between 99 and 100% for both genes, without the ambiguities. All TUB sequences are similar to *C.fioriniae* Acc.N. KX16773 while CAL sequences to *C.fioriniae* Acc.N. KJ954727 (tables 4 and 5).



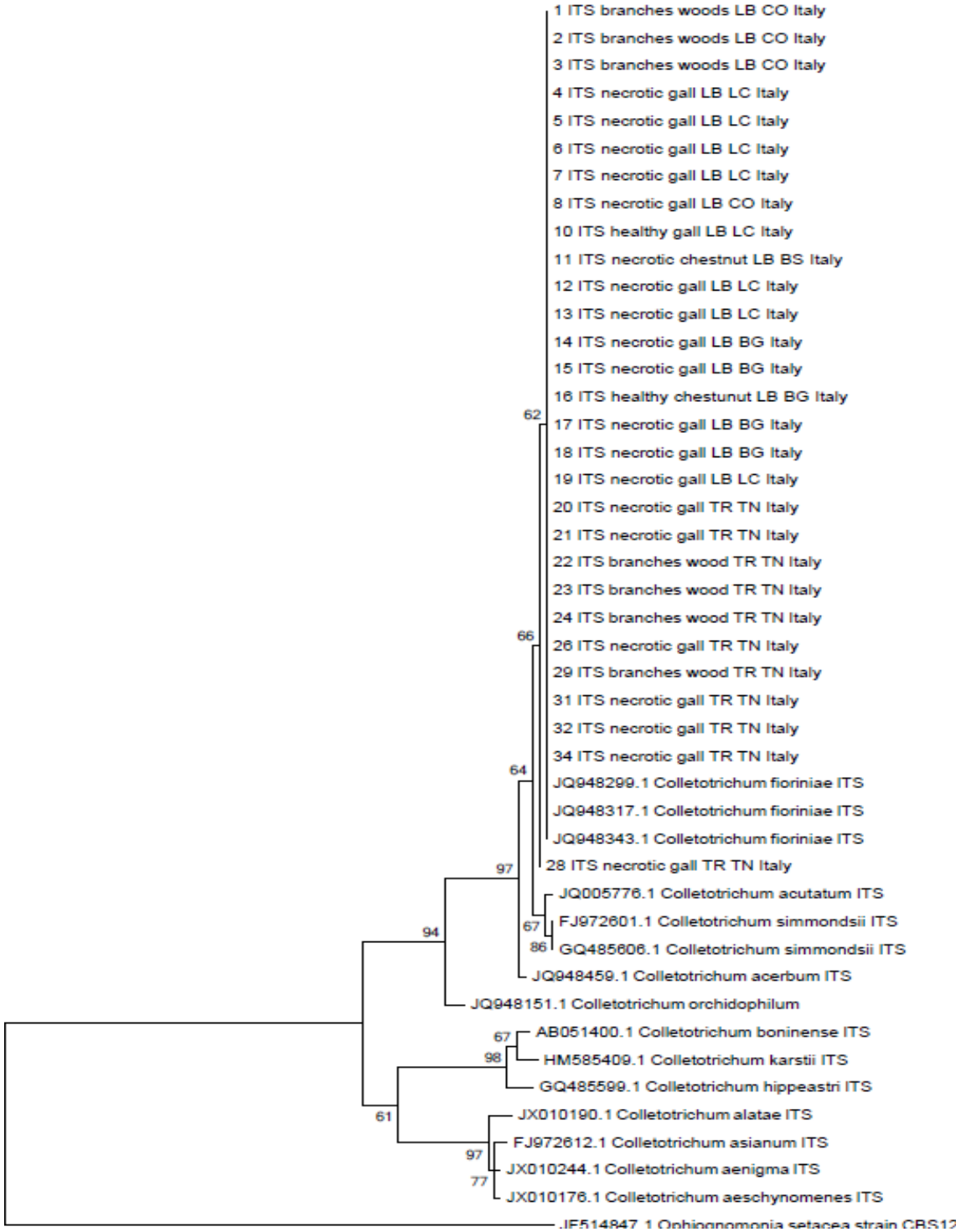
Phylogenetic tree based on rDNA TUB sequences showing the relationships among *Colletotrichum* spp. isolates. The tree was constructed by Neighbor-joining method, using the Jukes-Cantor-parameter pairwise distances. Topology was evaluated by bootstrap analysis (MEGA program, 1.000 replicates).

Ophiognomia setacea obtained in the GenBank was used as out group.



Phylogenetic tree based on rDNA CAL sequences showing the relationships among *Colletotrichum* spp. isolates. The tree was constructed by Neighbor-joining method, using the Jukes-Cantor-parameter pairwise distances. Topology was evaluated by bootstrap analysis (MEGA program, 1.000 replicates).

Ophiognomia setacea obtained in the GenBank was used as out group



Phylogenetic tree based on rDNA ITS sequences showing the relationships among *Colletotrichum* spp. isolates. The tree was constructed by Neighbor-joining method, using the Jukes-Cantor-parameter pairwise distances. Topology was evaluated by bootstrap analysis (MEGA program, 1.000 replicates).

Ophiognomia setacea obtained in the GenBank was used as out group.

Discussion

Molecular characterization of *Colletotrichum* isolates associated with galls and shoots bark of *C. sativa* resulted in the identification of *C. fioiriniae.* The result was strongly supported by the concordant data obtained from two protein-coding barcodes. Indeed, non-ribosomal barcodes are generally regarded as alternatives for discriminating fungi belonging to "difficult" genera, despite relevant drawbacks as poor yields and standardization of PCR reactions (Kress *et al.*, 2015). Instead, ITS, even if often regarded as a "universal barcode" for fungi, did not discriminate between the two close species *C. acutatum* and *C. fiorinae, a*s expectable for a ribosomal barcode used in this context (Damm *et al.,* 2012; Kress *et al.,* 2015).

This result is supported by numerous study carried out on *Colletotrichum acutatum*. Till now it is defined as a species complex (Baroncelli *et al.*, 2017), the morphological, physiological and molecular analysis recognized several intra-specific groups and these have been identified eight groups (A1–A8) based on rDNA-ITS and β-tubulin 2 (TUB) sequence analyses (Sreenivasaprasad and Talhinhas, 2005).

Nowadays, researches accepted 34 species divided in five clades, two of which are of narrow diversity (clades 3 and 4), while the other three contain at least eight species each, with clades 2 and 5 encompassing the largest genetic diversity within the *C. acutatum* species complex (Baroncelli *et al.,* 2017). The clade n.3 is represented by *C. fioriniae*. It is wellknown that a phylogenetic analysis based on a single gene, could not distinguish the species specificity in *Colletotrichum* complex (Mahdi Arzanlou *at al.*, 2015) and only the multigene phylogenetic analysis allow it (Cannon *et al.,* 2012; Damm *et al.,* 2010, 2012, 2014; Weir *et al.,* 2012).

Colletotrichum fioriniae (Marcelino & Gouli) R.G. (Shivas & Y.P. Tan, 2009) causes leaf and stem blight of *Acacia acuminata*, fruit rot of *Persea americana,* it was detected as endophyte in *Mangifera indica* and 28 other species of plants, and it is also entomopathogenic on elongate hemlock scale (*Fiorinia externa*) in the USA (Marcelino *et al.,* 2008). These authors confirmed the possible presence of the fungus as endophyte on hemlock.

From recorded data, we can state that *C. fioriniae* is present in Italy on *C.sativa*, confirming its extreme ability to colonize many cultivated and forest plants. To date, despite *C. fioriniae* causes anthracnose on different plant (Pszczółkowska *et al.*,2016; Pavel 2016), there is still no correlation between symptoms and the presence of *C. acutatum* on *C. sativa* even if, in a previous study

(Gaffuri *et al.,* 2016), it was observed a specific symptom of *Colletotrichum*, called “pink rot “ on chestnut nut (Gaffuri *et al.,* 2016). To overcome the problem of the identification of *Colletotrichum* in *C. sativa* without a proper symptomatology, on galls and shoots bark, the present study focused on the molecular characterization using ribosomal and non-ribosomal genes. Molecular analysis, performed by evaluating different genes, remains necessary because the different species of *Colletotrichum* can hardly be distinguished only morphologically.

The discovery of *C. fioriniae* open many question to be solved. *C. fioriniae* causes "Bitter rot" on post-harvest fruit and it may become an emerging problem for the apple fruit growing industry: could this species do the same for chestnut-nuts causing a new problem for chestnut cultivation? Data till now suggest the absence of a real problem but more investigation will be necessary. Furthermore, the discovery of a potential pathogen on chestnut in healthy tissue intrigues about its biological and ecological role. If not a potential pathogen for chestnut, could act against insect? Moreover could chestnut trees act as a sink of inoculums for other potential hosts?

Several host were reported to be affected by *Colletotrichum* spp. in Italy, among them *Olea* sp. (Schena *et al.*,2014), *Malus* sp.(Nodet et al., 2016), *Capsicum annuum* (Vitale *et al,* 2014) and other economical important species. The presence and the potential spread of the fungus in natural ecosystem as chestnut orchards and stands could affect the impact of the colligated disease in new areas or new host.

In this regard, it should be noted that, *C. acutatum* , in this study identified as *C.fioriniae*, was recently reported as an efficient parasite of the gall wasp in Italy (Gaffuri *et al.,* 2015).

Colletotrichum can be found as asymptomatic endophytes (Lima *et al.,* 2012) and mutualist or commensal endophytic associations between plants and members of the genus *Colletotrichum* have been reported (Meyer *et al.,* 2015). In the light of these observations it could be assumed that this fungus is naturally present in *C. sativa* as endophyte and that *C. fioriniae* is able to colonize the galls during their formation. Such a supposition would mean that larve of *D. kuriphilus* present in the galls may be infected with by *C.fioriniea* and this fungus could be have entomopathogenic action as already recognized.

Many questions still remain unanswered, therefore it is important to understand these interactions in order to foresee their effects and carry out more investigation in order to evaluate possible role in an epidemiological context.

Table 6

Colletotrichum species identification from branches wood, necrotic and healthy gall and *C.sativa* nuts by ITS gene analysis

Strain Name ITS	Host-Isolated from	Province-Region	Species identification	identity	GenBank ID
1_ITS	Branches wood	Como-Lombardy-Italy	[1]*C.acutatum*	100%	
2_ITS	Branches wood	Como-Lombardy-Italy	[1]*C.acutatum*	99%	
3_ITS	Branches wood	Como-Lombardy-Italy	[2]*C.fioriniae/C.acutatum*	100%	
4_ITS	Necrotic gall	Lecco-Lombardy-Italy	[3]*C.fioriniae*	100%	
5_ITS	Necrotic gall	Lecco-Lombardy-Italy	[4]*C.fioriniae/C.acutatum*	100%	
6_ITS	Necrotic gall	Lecco-Lombardy-Italy	[3]*C.fioriniae*	100%	
7_ITS	Necrotic gall	Lecco-Lombardy-Italy	[1]*C.acutatum*	100%	
8_ITS	Necrotic gall	Como-Lombardy-Italy	[1]*C.acutatum*	100%	
10_ITS	Healthy gall	Lecco-Lombardy-Italy	[3]*C.fioriniae*	100%	
11_ITS	Necrotic chestnut	Brescia-Lombardy-Italy	[1]*C.acutatum*	100%	
12_ITS	Necrotic gall	Lecco-Lombardy-Italy	[4]*C.fioriniae/C.acutatum*	100%	
13_ITS	Necrotic gall	Lecco-Lombardy-Italy	[5]*C.fioriniae/C.acutatum*	100%	
14_ITS	Necrotic gall	Bergamo-Lombardy-Italy	[8]*Glomerella acutata*	100%	
15-ITS	Necrotic gall	Bergamo-Lombardy-Italy	[3]*C.fioriniae*	100%	
16_ITS	Healthy chestnut	Bergamo-Lombardy-Italy	[2]*C.fioriniae/C.acutatum*	99%	
17_ITS	Necrotic gall	Bergamo-Lombardy-Italy	[8]*Glomerella acutata*	100%	
18_ITS	Necrotic gall	Bergamo-Lombardy-Italy	[6]*C.fioriniae/C.acutatum*	100%	
19-ITS	Necrotic gall	Lecco-Lombardy-Italy	[6]*C.fioriniae/C.acutatum*	100%	
20_ITS	Necrotic gall	Trento-Trentino-Italy	[6]*C.fioriniae/C.acutatum*	100%	
21_ITS	Necrotic gall	Trento-Trentino-Italy	[5]*C.fioriniae/C.acutatum*	100%	
22_ITS	Branches wood	Trento-Trentino-Italy	[5]*C.fioriniae/C.acutatum*	100%	
23_ITS	Branches wood	Trento-Trentino-Italy	[6]*C.fioriniae/C.acutatum*	100%	
24_ITS	Branches wood	Trento-Trentino-Italy	[7]*C.fioriniae/C.acutatum*	100%	
26_ITS	Necrotic gall	Trento-Trentino-Italy	[5]*C.fioriniae/C.acutatum*	100%	
28_ITS	Necrotic gall	Trento-Trentino-Italy	[5]*C.fioriniae/C.acutatum*	99%	
29_ITS	Branches wood	Trento-Trentino-Italy	[5]*C.fioriniae/C.acutatum*	100%	
31_ITS	Necrotic gall	Trento-Trentino-Italy	[8]*Glomerella acutata*	100%	
32_ITS	Necrotic gall	Trento-Trentino-Italy	[2]*C.fioriniae/C.acutatum*	100%	
34_ITS	Necrotic gall	Trento-Trentino-Italy	[1]*C.acutatum*	100%	

Note:
[1] % of identity with *C.acutatum* Acc.N. KX108948;
[2] the following species are equiprobable: *C.fioriniae Acc.N.* KY315935 and *C.acutatum* Acc.N. KY344751;
[3] % of identity with *C.fioriniae* Acc.N. KY271785,
[4] the following species are equiprobable: *C.fioriniae* Acc.N. KY315935 and *C.acutatum* Acc.N. KX078638;
[5] the following species are equiprobable: *C.fioriniae* Acc.N. KY271785 and *C.acutatum* Acc.N. KP064135;
[6] the following species are equiprobable: *C.fioriniae* Acc.N. KY271785 and *C.acutatum* Acc.N.KP064135;
[7] the following species are equiprobable*: C.fioriniae* Acc.N. KY271785 and *C.acutatum* Acc.N. KT823767
[8] % of identity with *Glomerella acutata* Acc.N. AB233348

Table 7

Colletotrichum species identification from branches wood, necrotic and healthy gall and *C.sativa* nuts by TUB gene analysis

Strain Name TUB	Host-Isolated from	Province-Region	Species identification	identity	GenBank ID
1_TUB	Branches wood	Como-Lombardy-Italy	*C.fioriniae	100%	
2_TUB	Branches wood	Como-Lombardy-Italy	*C.fioriniae	100%	
3_TUB	Branches wood	Como-Lombardy-Italy	*C.fioriniae	100%	
4_TUB	Necrotic gall	Lecco-Lombardy-Italy	*C.fioriniae	100%	
5_TUB	Necrotic gall	Lecco-Lombardy-Italy	*C.fioriniae	100%	
6_TUB	Necrotic gall	Lecco-Lombardy-Italy	*C.fioriniae	100%	
7_TUB	Necrotic gall	Lecco-Lombardy-Italy	*C.fioriniae	100%	
8_TUB	Necrotic gall	Como-Lombardy-Italy	*C.fioriniae	100%	
10_TUB	Healthy gall	Lecco-Lombardy-Italy	*C.fioriniae	99%	
11_TUB	Necrotic chestnut	Brescia-Lombardy-Italy	*C.fioriniae	100%	
12_TUB	Necrotic gall	Lecco-Lombardy-Italy	*C.fioriniae	100%	
13_TUB	Necrotic gall	Lecco-Lombardy-Italy	*C.fioriniae	100%	
14_TUB	Necrotic gall	Bergamo-Lombardy-Italy	*C.fioriniae	100%	
15-TUB	Necrotic gall	Bergamo-Lombardy-Italy	*C.fioriniae	100%	
16_TUB	Healthy chestnut	Bergamo-Lombardy-Italy	*C.fioriniae	100%	
17_TUB	Necrotic gall	Bergamo-Lombardy-Italy	*C.fioriniae	100%	
18_TUB	Necrotic gall	Bergamo-Lombardy-Italy	*C.fioriniae	100%	
19-TUB	Necrotic gall	Lecco-Lombardy-Italy	*C.fioriniae	100%	
20_TUB	Necrotic gall	Trento-Trentino-Italy	*C.fioriniae	100%	
21_TUB	Necrotic gall	Trento-Trentino-Italy	*C.fioriniae	100%	
22_TUB	Branches wood	Trento-Trentino-Italy	*C.fioriniae	99%	
23_TUB	Branches wood	Trento-Trentino-Italy	*C.fioriniae	99%	
24_TUB	Branches wood	Trento-Trentino-Italy	*C.fioriniae	99%	
26_TUB	Necrotic gall	Trento-Trentino-Italy	*C.fioriniae	99%	
28_TUB	Necrotic gall	Trento-Trentino-Italy	*C.fioriniae	99%	
29_TUB	Branches wood	Trento-Trentino-Italy	*C.fioriniae	100%	
31_TUB	Necrotic gall	Trento-Trentino-Italy	*C.fioriniae	99%	
32_TUB	Necrotic gall	Trento-Trentino-Italy	*C.fioriniae	99%	
34_TUB	Necrotic gall	Trento-Trentino-Italy	*C.fioriniae	99%	

Note: * % of identity with *C.fioriniae* Acc.N. KX16773

Table 8

Colletotrichum species identification from branches wood, necrotic and healthy gall and *C.sativa* nuts by CAL gene analysis

Strain Name CAL	Host-Isolated from	Province-Region	Species identification	identity	GenBank ID
1_CAL	Branches wood	Como-Lombardy-Italy	**C.fioriniae*	99%	
2_CAL	Branches wood	Como-Lombardy-Italy	**C.fioriniae*	99%	
3_CAL	Branches wood	Como-Lombardy-Italy	**C.fioriniae*	99%	
4_CAL	Necrotic gall	Lecco-Lombardy-Italy	**C.fioriniae*	99%	
5_CAL	Necrotic gall	Lecco-Lombardy-Italy	**C.fioriniae*	99%	
6_CAL	Necrotic gall	Lecco-Lombardy-Italy	**C.fioriniae*	99%	
7_CAL	Necrotic gall	Lecco-Lombardy-Italy	**C.fioriniae*	99%	
8_CAL	Necrotic gall	Como-Lombardy-Italy	**C.fioriniae*	99%	
10_CAL	Healthy gall	Lecco-Lombardy-Italy	**C.fioriniae*	99%	
11_CAL	Necrotic chestnut	Brescia-Lombardy-Italy	**C.fioriniae*	99%	
12_CAL	Necrotic gall	Lecco-Lombardy-Italy	**C.fioriniae*	99%	
13_CAL	Necrotic gall	Lecco-Lombardy-Italy	**C.fioriniae*	99%	
14_CAL	Necrotic gall	Bergamo-Lombardy-Italy	**C.fioriniae*	99%	
15-CAL	Necrotic gall	Bergamo-Lombardy-Italy	**C.fioriniae*	99%	
16_CAL	Healthy chestnut	Bergamo-Lombardy-Italy	**C.fioriniae*	99%	
17_CAL	Necrotic gall	Bergamo-Lombardy-Italy	**C.fioriniae*	99%	
18_CAL	Necrotic gall	Bergamo-Lombardy-Italy	**C.fioriniae*	99%	
19-CAL	Necrotic gall	Lecco-Lombardy-Italy	**C.fioriniae*	99%	
20_CAL	Necrotic gall	Trento-Trentino-Italy	**C.fioriniae*	99%	
21_CAL	Necrotic gall	Trento-Trentino-Italy	**C.fioriniae*	99%	
22_CAL	Branches wood	Trento-Trentino-Italy	**C.fioriniae*	99%	
23_CAL	Branches wood	Trento-Trentino-Italy	**C.fioriniae*	99%	
24_CAL	Branches wood	Trento-Trentino-Italy	**C.fioriniae*	99%	
26_CAL	Necrotic gall	Trento-Trentino-Italy	**C.fioriniae*	99%	
28_CAL	Necrotic gall	Trento-Trentino-Italy	**C.fioriniae*	99%	
29_CAL	Branches wood	Trento-Trentino-Italy	**C.fioriniae*	99%	
31_CAL	Necrotic gall	Trento-Trentino-Italy	**C.fioriniae*	99%	
32_CAL	Necrotic gall	Trento-Trentino-Italy	**C.fioriniae*	99%	
34_CAL	Necrotic gall	Trento-Trentino-Italy	**C.fioriniae*	99%	

Note:% of identity with *C.fioriniae* Acc.N. KJ954727

Note & Acknowledgements

Chapter 3 describes in detail the main issues emerging in the last year of my PhD. The article will be submitted for publication.

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Discussion

Chestnut tree (*Castanea sativa* Mill) is an economically important tree used for nuts and wood. Multiple management possibilities interesting from the production as well as the landscape and the biodiversity point of view are available. (Maltoni *et al.,*2012).

A correct agronomic management, such as the pruning forestry operations, is the minimum condition in order to preserve chestnut forests from deterioration and parasites attacks.

The major diseases which can affect the chestnut tree are “chestnut cancer”, a disease that together with the “ink disease” has threatened for years the survival of numerous plantations.

In the last ten years, however, the accidental introduction of an insect, *Dryocosmus kuriphilus* Yasumatsu, the chestnut gall wasp (ACGW), has caused production damage.

The wasp is a parasite native to Asia that may reduce yield in chestnut through both direct and indirect mechanisms (Battisti *et al.,* 2013). The galls can directly prevent the formation of the female flower when galls are formed on the apical buds of the shoots, which stops the growth and causes flower abortion (Battisti *et al.,* 2013). Moreover, the yield can be indirectly affected as a result of reduced leaf area, photosynthesis and tree biomass, and this effect can extend in the future years after the initial ACGW attack (Kato and Hijii, 1997).

To reduce the populations of galls wasp sits, the natural antagonist *Torymus sinensis* Kamijo was released. Therefore, chestnut populations in Lombardy was monitored to evaluate the effectiveness of this containment method. We observed the formation of necrotic galls from which we isolated different species and genera of fungi, including *Colletotrichum* sp.

Fungi comprise a vast variety of microorganisms and are numerically among the most abundant eukaryotes on Earth’s biosphere. The distribution of fungi among the various ecological niches of the biosphere seems to be infinite.

In 1990 the magnitude of fungal diversity was estimated 'conservatively' at 1.5 million specie (Hawksworth 1991; 2001), only less than a half has been merely described yet, and the actual range is properly estimated at 2.2 to 3.8 million (Hawksworth and Lücking, 2017).

Invasive (non-native) species are one of the great challenges facing the world, leading to great economic losses. The increase number of new species raises the likelihood of new interactions particularly between plants, microbes and insects.

Invasive species are alien (non-native) organisms that have been introduced into an area outside of their natural range, established self-sustaining populations and spread beyond their initial point of introduction, with deleterious impacts on the environment, the economy or human health (Kolar and Lodge, 2001). Invasive species may affect native species directly, through competition or predation,, or indirectly, by altering habitat or changing disease dynamics. Parasites may play a key role in mediating the impacts of biological invasions at any of the three phases: introduction, establishment or spread. Introduced alien hosts often have fewer parasite species and a lower prevalence of parasites than native hosts, providing them of a competitive advantage (enemy release; Mitchell and Power, 2003; Torchin *et al.,* 2003). Once introduction has occurred, parasite transmission may occur from native hosts to alien hosts, leading to an increase in infection of natives if aliens amplify transmission (Spillback; Kelly *et al.,* 2009; Mastisky and Veres, 2010) or a decrease in infection of natives if aliens reduce transmission (dilution; Paterson *et al.,* 2011; Poulin *et al.,* 2011). If alien hosts introduce new parasites, then these may be transmitted to native hosts, leading to the emergence of new disease in the natives (spillover or pathogen pollution; Daszak *et al.,* 2000; Taraschewski, 2006).

To threaten native hosts in a new locality, alien parasites must overcome the same barriers to introduction, establishment and spread as free-living aliens and, in addition, they must be able to switch from alien to native hosts. Lymbery *et al.,* (2014) proposed to use the terminology of “co-introduced” for those parasites which have entered a new area outside of their native range with an alien host, and “co-invader” for those parasites which have been co-introduced and then switched to native hosts .

In this contest some questions/ hypothesis arise: is *Colletotrichum* sp. found in the necrotic galls on chestnut tree to be considered a co-invader? Is this the reason why this is the first record of the fungus on chestnut tree and finally in Italy/Europe? In this case, is it to be regarded as a possible future pathogen for this plant?Otherwise, its record in the galls necrotic tissues could also suggest a potential role of the fungus in controlling *D. kuriphilus*: the insect is compelled to stop the development inside the gall as the necrosis destroy the gall itself.

Based on this assumption, it was thought to investigate the presence of *Colletotrichum* on other portions of the plant in particular healthy and necrotic gems, wood and galls: actually

Colletotrichum was present in different parts of the plant, both on healthy and necrotic portions, thus suggesting an endophytic behavior.

Colletotrichum is a common genus of fungi of a wide array of crops and non-cultivated plant species. Virtually every crop grown throughout the world is susceptible to one or more species of *Colletotrichum*. Species of the genus cause anthracnose spots and blights of aerial plant parts and post-harvest rots. The damage caused by *Colletotrichum* spp. extends to important food crops, including bananas, cassava and sorghum (Mill, 2001), grown by subsistence farmers in developing countries throughout the tropics and subtropics. It is particularly successful as a post-harvest pathogen because latent infections, which are initiated before harvest, do not become active until the fruit has been stored or appears on the market shelf. Up to 100% of the stored fruit can be lost as result of *Colletotrichum* disease (Prusky 1996; Wonsu *et al.,* 2016).

Anthracnose is a general term used to describe diseases that result in a wide range of symptoms including leaf spots, blotches or distortion, defoliation, shoot blight, twig cankers and dieback on many different deciduous trees and shrubs.

The disease produces great economic losses, estimated above 50% in crops of tamarillo, mango, blackberry, passion fruits (Afanador *et al.,* 2003), strawberry, cranberry, olives and other cultivated perennials in tropical regions (Onofre and Antoniazzi, 2014; Waller, 1992) and ornamental plant in different countries (Reed *et al.,* 1996; Wade *et al.,* 2001).

Colletotrichum species are also among the most commonly occurring foliar endophytes of terrestral plants and have been recorded from approximately 2,200 plant species (Farr and Rossman,2013).

As reporterd by Petrini (1991), endophytes infect living plant tissues without causing symptoms of disease. However, according to recent observations (Delaye *et al.,* 2013), many fungal endophytes are related to biotrophic and necrotrophic plant pathogens, thus suggesting that asymptomatic colonization is a balance of antagonism between the pathogen and the host (Devaraju and Satish 2010).

In the second part of the study (Chapter –II), the origin of the pink-coloured tissues observed in rotting chestnut nuts collected from soil in different orchards in Italy was investigated.

Morphological and molecular analysis confirmed the presence of *Colletotrichum acutatum* (J.H. Simmons) in affected fruits and inoculation tests corroborated the ability of the pathogen to colonize nuts.

The finding of this almost worldwide pathogen (Damm, Cannon, Woudenberg, & Crous, 2012) associated to a new symptomatology, characteristic pink coloration of nuts, confirms a possible risk related to its presence, because the endophytic isolates showed the same pathogenicity of those obtained from infected nuts.

Luckily, a very low level of infection has been recorded till now in all investigated chestnut stands (seven affected nuts of 15,000 damaged ones examined in 2015 in Trentino) (Gaffuri *et al.,* 2016).

This first discovery, opens a new questions about the role of *Colletotrichum* in the chestnut ecosystem, suggesting more controls on nuts and the investigation, monitoring and analysis of damage on stem and leaves.

It is knows that *Colletotrichum* sp. causes postharvest decays on many tropical, subtropical, and temperate fruits (Freeman *et al.,* 1996) and in particulary, *C.acutatum* has been found on peaches, apples, and pecans (Bernstein *et al.,* 1995) and on strawberries.

It is a common saprobe in citrus groves, invades dead and senescent leaves, twigs, and fruit and produces acervuli with abundant conidia on dead tissues of citrus. For these reasons, it is necessary to be able to determine if *Colletotrichum* whether even on *Castanea sativa*, could be considered a real threat for chestnut production and whether there is a possible role of *C. acutatum* in influencing the ecology and productivity of chestnut stands.

In the last part on my study, I focused on the molecular characterization of *Colletotrichum acutatum* isolates collected on leaves, gall, wood and nuts.

Currently, *t*he application of a polyphasic approach, including the analysis of geographical, ecological, morphological and genetic data is recommended, in order to establish a natural classification system for the genus Colletotrichum (Jayawardena *et al.,*2016). This genus has undergone frequent taxonomic changes in the past decades with the merging and addition of many species (Baroncelli *et al.,* 2017). Species concepts are still in a state of flux, however several major monophyletic clades, or species complexes, are now recognized (Cannon *et al.,* 2012).

The first name of *Colletotrichum* was introduced by Corda in 1831 and at the time of the first monographic treatment of *Colletotrichum* (Von Arx 1957), around 750 names existed (Cannon *et*

al. 2012). This high number of species unfortunately, is probably due a misidentification of *Colletotrichum* species, a frequent mistake that happens due to few distinctive morphological characters available for identification. A recent study provides an account of the 189 currently accepted species subdivided into 11 species complexes and 23 singleton species (Jayawardena *et al.,* 2016).

The multi genetic analysis is a method therefore necessary for the correct identification of the species. Twenty-nine strains were chosen for identification by phylogenetic analyses of multi-locus sequences, including the nuclear ribosomal internal transcribed spacer (ITS) region and the β-tubulin (TUB) and calmodulin (CAL) genes.

The amplified DNA with the ITS primers allowed only a partial identification of the isolates: only nine isolates were identified as *C.acutatum*, four as *C.fioriniae*, three as *Glomerella acutata.*

Glomerella acutata was described as the sexual morph of *Colletotrichum acutatum* (Guerber & Correll, 1997; 2001) as the product of mating experiments. Some related species are homothallic, including *Glomerella acutata* var. *fioriniae* (Marcelino *et al.,* 2008), later regarded as a separate species (*C. fioriniae,* Shivas & Tan 2009).

These results about ITS shows that a phylogenetic analysis based on a single gene, in particular the ITS, could not distinguish the species specificity in *Colletotrichum* complex (Mahdi Arzanlou at al., 2015); only the multigene phylogenetic analysis allow it (Cannon *et al.,* 2012; Damm *et al.,* 2010, 2012, 2014; Weir *et al.,* 2012).

The combination of ITS, TUB and CAL gene analysis help us to identify our isolates from galls, branches and chestnut on *Castanea sativa*, as *Colletotrichum fioriniae.*

Nevertheless this species, although reported in the Index Fungorum (http://www.indexfungorum.org/names/NamesRecord.asp?RecordID=515411) and in Mycobank (http://www.mycobank.org/BioloMICS.aspx?Table=Mycobank&Rec=559604&Fields=All) with the "Current name" *Colletotrichum fioriniae* (*Colletotrichum fioriniae* (Marcelino & Gouli) is discussed by Pennycook (2017) as follows:

"The widely used name " *Colletotrichum fioriniae*", published in 2009, is invalid; it is neither a valid new combination for " *Colletotrichum acutatum* var. *fioriniae*" (itself invalid) nor a valid new name, because no Latin diagnosis or description was presented or referenced. With the abolition of "dual nomenclature" (the separate nomenclatures of teleomorphs and anamorphs) under the current

International Code of Nomenclature...". Consequently he proposed a new combination based on *Glomerella acutata* var. *fioriniae* as the only validly published name for this *taxon*.

Once more we can say that the longstanding debate on the genus *Colletotrichum* and its species will never end.

The detection of *Colletotrichum fioriniae* on chestnut in healthy and necrotic portions of tissue makes us to argue about his biological and ecological role, both in the chestnut plant and in the insect: is this species to be regarded as a possible future pathogen for the plant? Is it to be regarded as potential BC agent in controlling *Dryocosmus kuriphilus*?

Future prospectives

The result of these researches have confirmed a substantial presence of C*olletotricum* in the investigated chestnut stands. The fungus was found as endophyte on branches and as colonizer on Asian wasp galls, sometimes also on dead *D. kuriphilus* larvae. The new symptoms of “Pink rot” suggests also its ability in colonizing nuts and producing some damages.

Anyway, until now characteristic symptoms related to the presence of this fungus, as those observed on other hosts, were never detected on chestnut trees and the damages on nuts seem really rare and not as dangerous as that caused by *Gnomoniopsis* sp.

The genetic analysis confirmed the difficult in characterizing the *Colletotricum* species but it permited to determine the investigated isolate as belongings to *C. fiorinae* complex. Therefore, isolates spreading in chestnut stands could be potential pathogens on several species. Chestnut trees could be a sink of inoculum for the spread of the pathogens in different contests.

Future investigation are needed to assess the effective absence of damages also on other species growing in the stands or nearby. Moreover, cross inoculation of the chestnut *Colletotricum* on other species could confirm this potential role.

The possible role as entomopathogens on *D. kuriphilus* need also to be confirmed even if , as like as for *Gnomoniopsis sp.*, it looks till no no so effective in checking the wasp.

New and interesting perspectives regards the possibilities to understand if this fungus is spreading as invasive or it is indigenoun and/or coevoluted component of chestnut ecosystem.

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Il mio immenso Grazie va poi ai compagni di avventura, alle amiche di zaino del "si escribes.. viajes" e ai "miei Conquistatori dell'inutile"...

Ora, sulla linea del traguardo posso guardare solo avanti, verso altre mete, altri obiettivi e altri viaggi poiché...
....Il viaggio non finisce mai. Solo i viaggiatori finiscono. E anche loro possono prolungarsi in memoria, in ricordo, in narrazione. Quando il viaggiatore si è seduto sulla sabbia della spiaggia e ha detto: "Non c'è altro da vedere", sapeva che non era vero. Bisogna vedere quel che non si è visto, vedere di nuovo quel che si è già visto, vedere in primavera quel che si è visto in estate, vedere di giorno quel che si è visto di notte, con il sole dove la prima volta pioveva, vedere le messi verdi, il frutto maturo, la pietra che ha cambiato posto, l'ombra che non c'era. Bisogna ritornare sui passi già dati, per ripeterli, e per tracciarvi a fianco nuovi cammini. Bisogna ricominciare il viaggio. Sempre.

Il viaggiatore ritorna subito.(José Saramago)