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The Asian Chestnut Gall Wasp in Italy: Surveys on its Native and Exotic Parasitoids as Well as on Chestnut Cultivar Susceptibility

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Authors' contributions

This work was carried out in collaboration among all authors. Author TP designed the study, performed the statistical analysis and wrote the protocol. Author MB wrote the first draft of the manuscript. Authors MB, FC, AT, TP, and RT carried out the field samplings. Authors MB, FC, AT and EG, managed the laboratory analyses. Authors MB and TP wrote the final draft and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

In the context of biological control against the Asian chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) in Italy, the aim of this study was to survey its native parasitoids as well as the exotic *Torymus sinensis* Kamijo which had been introduced and released as a biological control agent. Furthermore, both parasitism and pest susceptibility of local chestnut trees were investigated. Surveys were carried out in 2014 in the Municipality of Sambuca Pistoiese (Pistoia), in a chestnut forest which included three cultivars: Nerattino, Carpinese, and Pastinese. On a total of 35 trees, selected among the three cultivars, 24 current-year shoots were randomly collected and examined in the laboratory. After the damage by the cynipid had been assessed, leaf samples were used for molecular analyses. Galls were also collected on the 35 selected trees both for dissection and to rear parasitoid specimens in the laboratory. The three cultivars were confirmed by genetic tests. The Carpinese cultivar resulted to be the most damaged, with highest percentage of attacked shoots (84.38%). Moreover it was also the cultivar with the lower parasitism

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rate (about 16%). From reared galls seven parasitoid species emerged, all belonging to the superfamily of Chalcidoidea. Exception made for the non-native *T. sinensis*, the other six were native species associated to oak gall wasps. Native parasitoids were more scarcely present, while the exotic *T. sinensis* amounted for the 84.27% of all emerged parasitoids. Our results confirm how chestnut susceptibility to *D. kuriphilus* varies depending on the cultivar. Overall, the Nerattino cultivar resulted as the best among the three examined: it was subject to a less intense attack than the other cultivars, and the resulting smaller galls were more effectively exploited by parasitoids.

Keywords: Dryocosmus kuriphilus; Torymus sinensis; biological control; Cynipidae; chestnut growing.

ACRONYMS

ACGW : Asian chestnut gall wasp;
SSR : Simple sequence repeats;
UPGMA : Unweighted pair group method with arithmetic mean;

1. INTRODUCTION

A more profound awareness about the risks of chemical control products for plant protection has recently caused a higher demand for biological control measures. Although such a statement applies also to agriculture, this is especially true in forest management. Forests' higher complexity requires more sustainable measures to control phytophagous pests and natural strategies such as biological control are highly regarded. However, biological control is not totally exempt from side effects. In fact, some authors reported their doubts regarding the absence of negative consequences in classical biological control. Indeed, some papers report how these problems deeply affected, in the past, local invertebrate fauna, e.g. [1,2,3,4,5]. For this reason, the need for meticulous investigation is generally valued before – and after – the release of exotic antagonists outside their natural distribution range. Such precautions are key to assess the most accurate outcome when planning to use an exotic biological control agent (for reference see [6,7,8,9].

These issues are evident within the research on the Asian Chestnut Gall Wasp (ACGW) (*Dryocosmus kuriphilus* Yasumatsu) in Italian chestnut territories. The ACGW is one of the main pests of the genus *Castanea* (Mill.). It is native to China and, during the last century, it was accidentally introduced first to Japan and later to Korea, the U.S.A, and Europe.

In all new areas of introduction, the ACGW soon spread wherever chestnuts grow, becoming the major cause of chestnut forest decline and reduced yield. More specifically, Italy has the largest chestnut-producing area in Europe, with chestnut forests covering an area of about 850,000 hectares (including both coppice and nut-bearing stands). Here ACGW gall formation led to a severe decrease in the plant's growth rate, and in fruiting.

With the aim of controlling the ACGW in Italy the exotic parasitoid *Torymus sinensis* Kamijo, also from East Asia, was introduced and released in the field soon after the damage of the gall wasp had been reported in many Italian Regions. Indeed, in the new areas of introduction, *T. sinensis* could compete with the native antagonists which adapted to the ACGW as a new host. In this regard, it is worth mentioning how the introduction of *T. sinensis* in Japan affected the populations of the indigenous *Torymus beneficus* Yasumatsu & Kamijo [10]. Ten years after *T. sinensis* was released in Japan to control the ACGW, the populations of the exotic parasitoid had increased at the expenses of *T. beneficus* populations.

In Italy, these natural enemies include parasitoids which carry out their life cycle at the expenses of oak gall wasps. The association to the new host is facilitated by the presence of both chestnut trees and oaks in many Italian forests. Many investigations regarding the dynamics of ACGW native antagonists have been carried out in Italy, but the results which followed were quite dissimilar. In fact, while some papers reported negligible parasitism rates by native parasitoids, other authors observed far better effectiveness in controlling the ACGW, to the point of allowing some optimism about their potential [11,12,13,14,15,16,17]. The occurrence of competition with *T. sinensis* may negatively affect native parasitoids, by potentially decreasing their parasitism rates both on the ACGW and on native oak gall wasps. This could lead, in the worst-case scenario, to more damage on oaks caused by exploding populations of native gall wasps. Besides the role of parasitoids, the selection of less susceptible chestnut cultivars may be another approach to limit the damage of the ACGW. In Japan, studies have shown how chestnut susceptibility varies depending on the cultivar. Moreover, they verified that more tolerant cultivar had both fewer ACGW attacks and an increased tendency to induce ACGW larval mortality [18]. Several trials were carried out in Japan to identify the most tolerant cultivars and a total of four cultivars were chosen and selected to be later placed in the market: Tsukuba, Tanzawa, Ibuki, and Ishizuchi [18]. Also in Italy, surveys have been launched to discover the most tolerant cultivars of *Castanea sativa* (Mill.), the Italian chestnut. Preliminary results showed that, even though all the observed cultivars are attacked by the ACGW, some differences exist in symptoms and ACGW oviposition preferences [15,19].

The main object of the present study was to assess occurrence and activity of *T. sinensis* as well as of native parasitoids in Italy, natural enemies of the ACGW, taking into account different chestnut cultivars. This goal is crucial to verify both effectiveness and side effects of the use of *T. sinensis* as a biological control agent against the ACGW. Finally, investigating chestnut cultivar susceptibility in Italy may help gathering useful data for the management of chestnut forests, especially when planning new plantations or if old ones need some restoration. More specifically, if the less susceptible cultivars also produce good quality fruits, they may be chosen in the place of the more heavily attacked by the ACGW.

2. MATERIALS AND METHODS

2.1 Study Area

Surveys were carried out in 2014 in the Municipality of Sambuca Pistoiese (Pistoia), in chestnut forest at an altitude of 700 m (44.064926N, 10.923378E), on the northern hillside of Mount Pidocchina. Climate is typical of sub-mountain Tuscany, with the highest mean maximum temperatures in July (25.3°C) and lowest mean minimum temperatures in January (-0.08°C) (climate-data.org). Highest monthly rainfall occurs in November (120 mm), while the lowest one is recorded in July (45 mm) (climate-data.org). The *C. sativa* forest was infested by the ACGW, included three different cultivars (Nerattino, Carpinese, and Pastinese), and was surrounded by woods mainly composed by oak trees. Finally, starting in 2012, multiple *T. sinensis* introductions were carried out in our study area which happened to be one of the release sites within the regional Biocontrol program against the ACGW.

2.2 Sampling of Chestnut Shoots and Laboratory Analyses

Based on the information provided by the local chestnut growers we selected 35 trees from the three aforementioned cultivars as well as from a forth one known as Ceppa. On May 22nd, 2014 a total of 24 current-year shoots were randomly collected for each tree. Every shoot was labeled according to the tree source and then brought to the DAGRI laboratories, University of Florence, Italy) for further analysis. First, we established the presence/absence of ACGW galls on every shoot for each chestnut tree, then leaf samples of the selected trees were used for molecular analyses to verify which cultivar they belonged to.

2.3 DNA Isolation, SSR Amplification and Analysis

DNA was extracted from 50-80 mg of fresh leaves according to Qiagen DNeasy^{TM} Plant mini Kit protocol. Information on microsatellites, also known as simple sequence repeats (SSR), was obtained from fifteen genomic SSRs developed in *C. sativa* Mill [20,21].

PCR amplification of SSR loci was carried out using a reaction mixture (20 µl), consisting of 2 µl 10X buffer (10 mM Tris-HCl, pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, 0.5 µM of each primer, 200µM dNTP, 0.5 U *Taq*-DNA polymerase (AmpliTaq Gold polymerase, Applied-Biosystems) and 50 ng template DNA, were carried out on a GeneAmp 2700 Thermal Cycler (Applied Biosystems).

Amplification cycles consisted of an initial step of 7 min at 96°C, followed by 40 cycles of 45 s at 95°C, 45 s at the annealing temperature of each primer, 1 min at 72°C. The amplification product was run on an ABI PRISM 3100 DNA sequencer. Allele scoring was performed using the GeneScan 3.5 and Genotyper 3.7 softwares (Applied Biosystems). MEGA 3 Software was used to create a dendrogram from a distance matrix. The program calculates a similarity matrix (only for option a), transforms similarity coefficients into distances, and makes a clustering using the Unweighted Pair Group Method with Arithmetic mean (UPGMA).

2.4 Sampling and Rearing of Galls

Galls were collected on the 35 selected chestnut trees during two samplings which were carried out on May 22nd, 2014 and May 26th, 2014. During each sampling 16 galls were collected at random on each tree within three meters of height from the ground, using a long reach pruner. Three quarters of the galls (12 for each tree sampling) were used for laboratory observations. Each gall was labelled, dissected, and observed under a stereo microscope to record the total number of cells per gall as well as the number of parasitized cells. The remaining galls (four for each tree sampling) were labelled and stored in confined environment at room temperature (ranging from 15 to 25°C) and placed individually inside clear plastic cups covered with a fine mesh. Galls were checked every three days for ACGW and parasitoid emergence. For the adult parasitoids' identification, we used an unpublished taxonomic key compiled by R. R. Askew (Manchester, UK), which is a basic identification tool on species level used for decades in the research of oak gall wasps' parasitoid communities. To assess parasitism rates only the dissected galls were taken into account, since the resulting data are more reliable than recording parasitoid emergence [17]. Parasitism rate was evaluated applying the following formula:

Parasitism rate = n. of parasitized cells per cultivar / total n. of cells per cultivar *100

2.5 Statistical Analysis

The percentage of attacked shoots and the percentage of parasitized cells were compared using a squared *chi* test, applying the Kimball formula [22] for the partition of the degrees of freedom. For each cultivar, the mean number of cells per gall was compared with the analysis of variance. Tukey's test was used for the comparison of means.

3. RESULTS AND DISCUSSION

The molecular tests showed that all sampled chestnut trees, except one, belonged to cultivars Carpinese, Nerattino, and Pastinese (Fig. 1). Indeed, the trees expected to be belonging to the Ceppa cultivar were in fact included, for the most part, among the other three. Thus, trees were assigned to one of the three main cultivars, based on molecular results, and their respective data was allocated accordingly. Molecular analysis was central in confirming the identity of each chestnut tree, as the initial information provided by the local chestnut growers resulted to be only partially correct (Fig. 1). This suggests that without an accurate genetic survey of the host plants, observations on the ACGW and its parasitoids may lead to erroneous results.

The three sampled cultivars were not equally attacked by the ACGW ($\chi2$= 51,084, df=2; P<.001). in fact, the Carpinese cultivar was the most damaged, with highest percentage of attacked shoots (84.38%), while Nerattino was the least attacked (53%), followed by Pastinese (66.03%) (Fig. 2). Even the mean number of cells per gall (therefore the average amount of ACGW specimens) significantly varied among the three different cultivars (Analysis of Variance, df=2, F=42.350, P<.001). In fact, Nerattino galls had on average fewer cells (1.91±0.09) compared with Pastinese (2.21±0.08) and Carpinese (2.74±0.08) cultivars.

As far as parasitoids are concerned, a significantly inferior parasitism rate of sampled galls was recorded for the Carpinese chestnut trees (about 16%), when compared with the other two cultivars (Table 1), which had parasitism rates higher over 19% (Table 1). From reared ACGW galls seven parasitoid species emerged, all belonging to the superfamily of Chalcidoidea (Table 2); exception made for the non-native *T. sinensis*, the other six were native species associated to oak gall wasps, already recorded in Tuscany as adapted to the ACGW [15,16,17]. However, we observed a limited presence of native parasitoids, while the exotic *T. sinensis* amounted for the 84.27% of all emerged parasitoids (Table 2).

Our results confirm how chestnut susceptibility to the ACGW varies depending on the cultivar, as already observed by other authors [15,19]. In fact, the three cultivars examined in this study showed a different susceptibility to the gall maker, as the Nerattino cultivar was less affected by *D. kuriphilus* than both Carpinese and Pastinese cultivars. Indeed, this cultivar had a lower percentage of attacked shoots and a lower number of cells per gall. Besides, also the parasitism turned out to be different, as the galls of the Nerattino cultivar, as well as those from Pastinese, had a higher percentage of parasitized cells. This result is in agreement with the statement by Panzavolta et al. [17], namely, that parasitoids attack smaller sized galls more efficiently, succeeding, in these galls, in parasitizing a larger number of cells. In fact, since the number of cells per gall is related to the size of the galls themselves [15] we can state that the Nerattino cultivar was the one with the smallest galls.

Native parasitoids were found to be present in the galls, as was to be expected given the proximity of the study area to an oak forest. However, their presence was found to be rather limited, while the parasitism by *T. sinensis* was

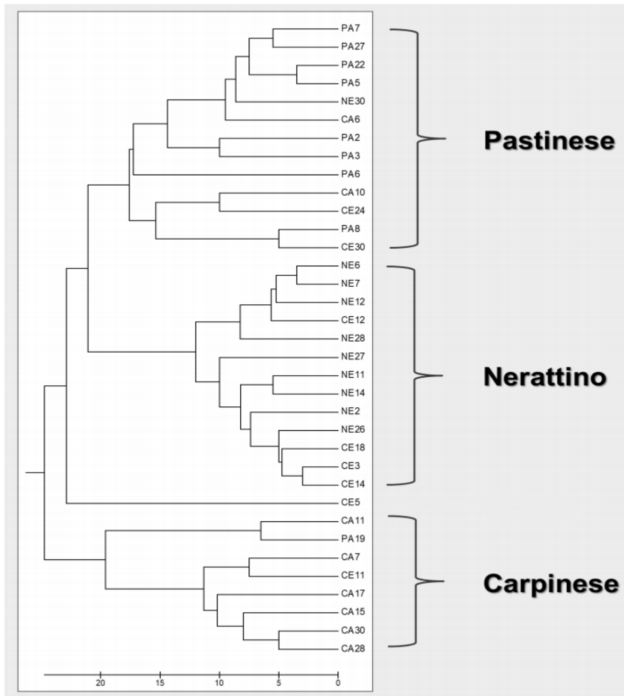

Fig. 1. Dendrogram designed according to genetic distances of the sampled chestnut trees: three major groups can be observed, namely Pastinese, Nerattino, and Carpinese cultivars, sample names show the expected identity according to local chestnut growers (PA=Pastinese; CA=Carrarese; CE=Ceppa; NE=Nerattino)

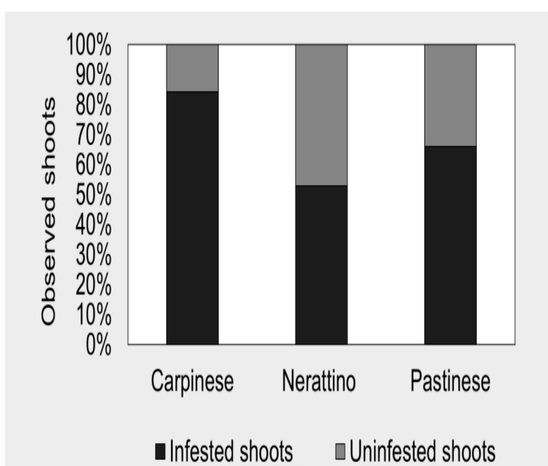

Fig. 2. Percentage of attacked shoots among the total sampled shoots, divided among the three chestnut cultivars of our study area (Municipality of Sambuca Pistoiese)

Table 1. Parasitism rates recorded on *D. kuriphilus* galls sampled in the Municipality of Sambuca Pistoiese (Pistoia) and results of the statistical analysis

Chestnut cultivar		**Parasitism rate (%)**	
Carpinese		16.42	
Nerattino		22.61	
Pastinese		19.17	
χ2 and Kimball's tests results			
Comparisons parasitism rates	**χ2**	**df**	**P**
Nerattino vs Pastinese	3.711	1	ns
Carpinese vs (Nerattino + Pastinese)	10.821	1	<.01
Total	14.532	2	<.001

Table 2. Parasitoids emerged from *D. kuriphilus* galls sampled in the Municipality of Sambuca Pistoiese (Pistoia)

Parasitoid species (Hymenoptera, Chalcidoidea)	**Chestnut cultivars**			
	Carpinese	**Nerattino**	**Pastinese**	**Total**
Mesopolobus sericeus (Forster) (Pteromalidae)	0	0	1	2
Mesopolobus tibialis (Westwood) (Pteromalidae)	0	0	1	1
Sycophila biguttata (Swederus) (Eurytomidae)	0	0	0	1
Eupelmus urozonus Dalman (Eupelmidae)	5	2	0	7
Torymus flavipes(Walker) (Torymidae)	1	4	0	5
Torymus auratus (Muller) (Torymidae)	0	3	1	4
Torymus sinensis (Torymidae)	27	42	38	107
Total	33	51	41	127

definitely more important, especially considering that the releases of the non-native parasitoid in the area took place only two years earlier than our observations. However, the presence of two species of the genus *Torymus*, *T. flavipes* and *T. auratus*, must be taken into particular consideration since, *T. sinensis* could cause genetic changes in populations of similar species through hybridization phenomena, as already observed in Japan for *T. sinensis* and *T. beneficus* [23], although not yet observed in Italy [24,25].

4. CONCLUSION

Finally, Nerattino seems to be the less susceptible cultivar among the three examined. In fact, it is subject to a less intense attack than the other cultivars and the resulting galls do not reach the average size observed in Pastinese and Carpinese. Besides, smaller galls are more efficiently exploited by the ACGW parasitoids; this translates into higher parasitism rates and, therefore, both native parasitoids and *T. sinensis* may control ACGW more effectively.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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