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Published version:

DOI:10.1007/s10526-015-9676-1

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Non-target host risk assessment for the parasitoid *Torymus sinensis*

Abstract

Torymus sinensis Kamijo (Hymenoptera: Torymidae) has been released throughout Italy for biological control of the chestnut gall wasp. In response to concern about non-target impacts associated with the introduction of this exotic biological control agent, this study aimed at investigating *T. sinensis*'s host range. In total, 1,371 non-target galls were collected in north-central Italy in a two-year period, representing nine different species. Collections were carried out on common oak, downy oak, sessile oak, Turkey oak, and wild rose.

A total of five native torymid species were recorded from the non-target galls (*Megastigmus dorsalis*, *Torymus affinis*, *T. auratus*, *T. flavipes*, and *T. geranii*), and three ♂♂ *T. sinensis* individuals emerged from *Biorhiza pallida* galls collected in the field. Under controlled conditions, most of the non-target galls tested were not suitable hosts for oviposition. *T. sinensis* females only laid eggs on *Andricus curvator*. In olfactometer bioassays, higher numbers of *T. sinensis* females showed more interest to the chestnut galls compared to non-target hosts. This data highlights how *T. sinensis* has a broader ecological host range than reported in the literature and that it is attracted by non-target hosts other than *D. kuriphilus*.

Keywords: *Torymus sinensis*, risk assessment, host specificity, chestnut gall, oak gall

Introduction

Biological control of arthropod pests, weeds and plant diseases has been practiced for centuries. It is a cost-effective, environmentally friendly approach to resolve pest problems in terrestrial and aquatic ecosystems. Classical biological control, in particular, involves selecting natural enemies of invasive species in their native range and releasing them in a recently invaded environment. It can assure lasting, highly selective, and effective pest control (McEvoy 1996), but in addition to providing a long-term benefit, the dispersal and permanent establishment of a beneficial insect for biological control leads to an irreversible situation with the potential to cause negative consequences to species other than the target pest (Andreassen et al. 2009; Brodeur 2012).

Prey/host specificity appears to be one of the most variable biological traits of biocontrol agents; natural enemies currently used in biological control programmes may show various degrees of specificity, ranging from organisms having a narrow host range restricted to a single species or genus to those with a wide spectrum of potential hosts covering several orders, classes or even kingdoms. Specificity primarily establishes the intrinsic potential of a given species to become an efficient safe natural enemy of a target pest (Brodeur 2012). Risks to non-target species from biological control programmes were already noted in the 1980s; issues concern the risks, if any, that biological control agents introduced to new countries may pose, causing a decline in species that are not the target pest (Howarth, 1991; Strand and Obricky 1996; Brodeur 2012).

Torymus sinensis Kamijo is a biological control agent of the chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu that has been distributed in Japan (1975), the USA (in the late 1970s), Italy (2005), and France (2011) (Moriya et al. 1989; Cooper and Rieske 2007; Quacchia et al. 2008; Borowiec et al. 2014). It is reported in the literature as univoltine like its host, predominantly reproducing amphigonically, even if recent evidence proved it may exhibit a prolonged diapause mainly as late instar larva. After emergence, that takes place in early spring, and mating, the female lays eggs inside the larval chamber of newly formed galls, usually one egg per host larva. After hatching the larva feeds ectoparasitically on the host larva, and it pupates in the host larval chamber during winter (Moriya et al., 1990; Ferracini et al. 2015).

The introduction of this parasitoid is widely known as one of the typical successful cases of classical biological control in Japan and Italy too (Moriya et al. 2003; Ferracini et al. 2015); however, the risks of the agent concerning potential negative effects on non-target native gall makers mainly present on oaks (*Quercus* spp.) and closely related parasitoids have never been evaluated thoroughly. Murakami et al. (1977) reported *T. sinensis*, among the Chinese parasitoids, as the only species host specific and synchronous with the chestnut gall wasp, but we currently know little about its host range and host specificity in its native or introduced ranges. A few limited host range tests were carried out under laboratory conditions with alternative host galls from *Mikiola fagi* Hartig (Diptera: Cecidomyiidae) , and on asexual generation of the oak gall wasps *Cynips quercusfolii* L. and *Andricus kollari* Hartig (Hymenoptera: Cynipidae) in 2004 (Quacchia et al. 2008), but no oviposition ever occurred. More preliminary tests with seven oak gall species [*A. crispator* Tschek, *A. curvator* Hartig, *A. cydoniae* Giraud, *A. grossulariae* Giraud, *A. multiplicatus* Giraud, *Biorhiza pallida* Olivier, and *D. cerriphilus* Giraud (Hymenoptera: Cynipidae)] were also performed in 2014, supporting the specificity thesis further (Quacchia et al. 2014).

In response to concern about non-target impacts associated with the introduction of this exotic biological control agent, the EFSA Panel on Plant Health established a new alternative host species list for testing the host-specificity of *T. sinensis*, comprising galls which may be more susceptible to attack during the period that females are searching for hosts, such as *A. curvator* sexual generation; *A. cydoniae* sexual generation; *A. grossulariae* sexual generation; *A. inflator* sexual generation; *A. lucidus* sexual generation; *A. multiplicatus* sexual generation; *B. pallida* sexual generation; *D. cerriphilus* sexual and asexual generation; *Neuroterus quercusbaccarum* (L.) sexual generation) (EFSA Panel On Plant Health 2010).

The present study aimed at investigating *T. sinensis*'s host range, with particular regard to the oak galls species listed by EFSA Panel On Plant Health (2010). No choice trials were carried out applying recently-developed protocols for host/prey range testing, considering the hypothesis that species most closely related taxonomically and ecologically to the target are more likely to be utilized as hosts by the biological control agent being tested (Kuhlmann et al. 2006; van Lenteren et al. 2006). Furthermore, olfactometer bioassays were performed to assess the attractiveness of volatiles for *T. sinensis* females; experiments were conducted to test the response of the parasitoid comparing chestnut galls, reported in literature as the only target host, *versus* non target oak galls.

Materials and methods

<u>Insect</u>

All *T. sinensis* used in the trials emerged from parasitized chestnut galls randomly collected, by hand from low branches (from ground level to 2 m high) and with the aid of lopping shears from the medium-high tree crown (from 2 to 5 m high), once a year, in winter, both in 2013 and 2014 in chestnut orchards in Piemonte region, where the parasitoid was first released in 2005 and then successfully established. The galls were kept in cardboard boxes outdoors from January to April until emergence of the parasitoids (Tmin=-1.9, Tmax=16.3°C, RHmin=67.8=, RHmax=78.8% in 2013; Tmin=0.8°C, Tmax=19.3°C, RHmin=64.5=, RHmax=81.1% in 2014) . Newly emerged females were fed every 48 h with drops of honey on cardboard and individually kept in glass tubes (120 mm in height by 18 mm in diameter), with no previous contact with a host, in a climatic chamber at 15 ±1 °C, 60±5% RH, and a photoperiod of 16:8 (L:D) h.

<u>Gall collection</u>

Non-target galls were collected in 4 regions (2 sites per region): Liguria [Borzonasca (GE) 44°26’01.6’’N 9°23’45.9’’E, Sassello (SV) 44°29’33.7’’N 8°33’17.9’’E], Piemonte [Molare (AL) 44°34’40.2’’N 8°36’10.5’’E, Pianfei (CN) 44°19’41.2’’N 7°40’58.9’’E], Toscana [Marradi (FI) 44°04’53.1’’N 11°38’17.8’’E, Piazza al Serchio (LU) 44°10’31.8’’N 10°17’14.9’’E], Valle d’Aosta [Arnad (AO) 45°38’22.7’’N 7°43’41.8’’E Perloz 45°36’53.9’’N 7°48’24.4’’E] (Figure 1). Collections were carried out on common oak (*Quercus robur* L.), downy oak (*Q. pubescens* Willdenow), sessile oak (*Q. petraea* (Mattuschka) Lieblein), Turkey oak (*Q. cerris* L.), and wild rose (*Rosa* spp. L.). The selection of non-target oak hosts was based on the species list for host-specificity testing established by EFSA Panel On Plant Health (2010), and host galls from the genus *Rosa*, since their abundance in the wood, were tested as well as suggested by Gibbs et al. (2011). Investigations were carried out over a 2-year period (2013–2014) in all regions and in the same sites. Withered galls were collected in January-February in order to verify if *T. sinensis* may emerge from non-target hosts, and fresh galls were collected in April-May (during *T. sinensis* emergence) both on non-target hosts and chestnut trees (used as a control), to perform oviposition trials and olfactometer bioassays under controlled conditions.

Sampled trees and shrubs were located in mixed forests close to infested chestnut stands where *T. sinensis* was previously released in order to evaluate a potential shift from chestnut to non-target hosts. In the Table 1 the years of release and the coordinates of *T. sinensis* release points are provided. Collections were made by hand from low branches (from ground level to 2 m high) and with the aid of lopping shears from the medium-high tree crown (from 2 to 5 m high). Galls were stored in plastic bags, transferred immediately to the laboratory, and identified using voucher specimens deposited at the DISAFA-Entomology laboratory.

Withered galls were individually isolated in plastic containers (70 mm in height by 55 mm in diameter) with a fine-gauze lid, and stored outdoors within 24 hrs from collection. Containers were checked weekly, and then daily after the first parasitoid emerged. Fresh galls were kept in climatic chamber at 15 ±1 °C, 60±5% RH, and a photoperiod of 16:8 (L:D) h and used in the trials within 24 hrs from collection. Containers were kept outdoors from January to December until emergence of the parasitoids (Tmin=-1.9, Tmax=29.1°C, RHmin=61.7%=, RHmax=83.4% in 2013; Tmin=0.8°C, Tmax=26.3°C, RHmin=63.0%=, RHmax=89.7% in 2014) .

No choice oviposition trials

These tests, based on the 'no choice black box test' (van Lenteren et al. 2006), aimed to test *T. sinensis*'s host range, investigating the parasitoid's ability to develop in non-target hosts. No choice exposures to non-target hosts were conducted in an enclosed arena in order to maximise the likelihood of non-target attack. Four fresh oak gall species included in the EFSA list were tested in 2013 (*A. curvator*, *A. grossulariae*, *B. pallida*, and *N. quercusbaccarum*), and only two in 2014 (*A. curvator*, and *B. pallida*).

A single fresh non-target gall was offered to a 6-d-old naïve *T. sinensis* female placed on a wet filter paper inside a Petri dish (diameter, 10 cm) in order to obtain data on its behaviour in relation to parasitism, with a minimum of 20 replications for each gall species. Observations were performed using a stereomicroscope for 1 hour. Three behavioural sequences were recorded: host location, defined as a walk on the gall locating the host through vibrotaxis, attempted oviposition, defined as the attempted insertion of the ovipositor, and oviposition, defined as successful insertion of the ovipositor followed by the pumping action of the abdomen.

At the end of the observation the female was then removed from the arena and individually isolated in a Petri dish containing a fresh unparasitised chestnut gall as control, and the three behavioural sequences were recorded following the same procedure described above. All the tested galls were individually stored in glass tubes (120 mm in height by 18 mm in diameter), and then dissected with the aid of a scalpel using a stereomicroscope. Since eggs may have escaped detection, galls were stored in a climatic chamber at 24±2°C, 50±10% RH, and a photoperiod of 16:8 (L:D) h for 10 days to ease the detection of the parasitoid at larval stage. All the trials were performed in laboratory conditions. To avoid any influence in the behaviour of the parasitoid, chestnut galls were collected in Alto Adige region in a site with no presence of *T. sinensis*. On the contrary, since during collection it was not possible to detect previously parasitised galls (e.g. by visual inspection), oak galls were discarded after the trials if any native parasitoid larva was identified by molecular analysis after dissection.

Olfactometer bioassays

In the olfactometer bioassays, 6-d-old *T. sinensis* females were used to assess their olfactory responses to the odours of the chestnut gall (used as a control) and 6 non-target galls (*A. curvator*, *A. cydoniae*, *A. grossulariae*, *A. multiplicatus*, *B. pallida*, *N. quercusbaccarum*) as alternative hosts.

Before the trials, the insects were individually kept at room temperature without any host in a glass tube for 18 h with a humid cotton cap and microdrops of honey to acclimate the wasps to the experimental conditions. The bioassays were carried out in a horizontal Y-shaped Pyrex tube following the procedure described for another wasp, *Necremnus* spp. (Ferracini et al. 2012). The air flow was provided by an air pump (Air 275R, Sera, Heinsberg, Germany) and then filtered in an activated CO2 filter, regulated with a flowmeter at 2.5 liters/min (EK-2NRK, Comer, Bologna, Italy) and humidified in a 1-liter water bubbler half filled with deionized water. After the air flow was established, a single parasitoid female was introduced into the entry arm. Each female was observed until she had moved at least 2 cm up one of the side arms or until 10 min had elapsed. For each test the same odour sources were used while concerning the wasps, a female was evaluated only once to prevent any behaviour conditioned by experience. The odour sources chosen by females that responded were recorded. Thirty responses were recorded for each pair of odour sources. After testing five females, the odour sources were switched between the left-hand and right-hand side arms to minimize any spatial effect on choices. The Y-tube and cameras were cleaned with mild soap and alcohol (70%v) and sterilized in an autoclave at 120°C for 20 min. The olfactory bioassays were conducted at 24±2°C, 50±10% RH, and 250±10 lux.

<u>Parasitoid identification</u>

Among all the parasitoids emerged from non-target hosts, only the torymid species were morphologically identified using specific dichotomous keys (Kamijo 1982; de Vere Graham and Gijswijt 1998) and by comparison with voucher specimens deposited at the DISAFA-Entomology laboratory. Doubtful species and larvae recorded in dissected galls in the no choice oviposition trials were submitted to DNA extraction and then sequenced for the cytochrome oxidase I (COI) gene following Kaartinen et al. (2010).

<u>Statistical analyses</u>

In the behavioural trials the numbers of times that *T. sinensis* females engaged in three types of behaviour (host location, attempted oviposition, and oviposition) were recorded, and means were analyzed for each non-target gall to *D. kuriphilus* gall (as control) by paired t-tests for dependent samples. In the olfactory bioassays, the responses of parasitoid females were analysed by a chi-square test. The null hypothesis was that parasitoid females had a 50:50 distribution across the two odour sources. All analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL).

Results

In total, 1,371 non-target galls were collected over the 2-year period, corresponding to 4 different genera: *Andricus*, *Biorhiza*, *Diplolepis,* and *Neuroterus* (Table 2). The galls found most frequently were the sexual generations of *B. pallida* (856), while only 3 galls from *A. grossulariae* and 1 gall from *A. lucidus* were recorded. A total of 707 native torymid specimens emerged from the isolated galls, belonging to 5 species: *Megastigmus dorsalis*, *Torymus affinis*, *T. auratus*, *T. flavipes*, and *T. geranii* (Table 3). The most frequent species was *T. flavipes* (381 specimens), while *M. dorsalis* (6 specimens), and *T. geranii* (3 specimens) were recorded sporadically. *B. pallida* galls proved to be parasitized by all the parasitoid species recorded, except for *M. dorsalis*, which was recorded emerging from galls of *A. cydoniae*, *A. lucidus*, and *A. multiplicatus*. *T. geranii* emerged only from *B. pallida* galls, and the only species recorded emerging from *D. rosae* was *T. auratus*.

In addition to native torymid species, in 2013 a total of 3 ♂♂ *T. sinensis* individuals emerged from non-target *B. pallida* galls collected in the Piemonte region in both surveyed sites (2 ♂♂ from Pianfei and 1 ♂ from Molare). The cytochrome oxidase I gene obtained from the specimens submitted to molecular identification was sequenced and sequences were compared with those in the National Center for Biotechnology Information (NCBI) sequence database. In all cases, a minimum of 99 % similarity with *T. sinensis*-related sequences was observed.

No other emergence of the exotic parasitoid was recorded for the other non-target oak galls nor for *D. rosae* during the surveyed period (Table 3). All the torymid species emerged from the withered non-target galls were collected between April and May, depending on the site.

<u>No choice oviposition trials</u>

In the no choice oviposition trials all the behavioural traits recorded on non-target and target hosts are reported in Table 4. In the close confinement imposed by the experimental design, *T. sinensis* females responded to all non-target and target species by locating and investigating the hosts. The number of host location events was significantly lower for *A. curvator* both in 2013 and 2014 (t test=-4.59; df=29; P<0.001 in 2013; t test=-2.99; df=29; P=0.008 in 2014), and for *A. grossulariae* (t test=-3.14; df=19; P=0.005), and *N. quercusbaccarum* (t test=-4.36; df=19; P<0.001) compared to the control, while for *B. pallida* no significant differences were revealed (t-test=-0.82; df=19; P=0.42 in 2013; t-test=-1.18; df=19; P=0.25 in 2014). Attempts of oviposition were observed when the parasitoid was offered *B. pallida* galls both in 2013 and in 2014 (t-test=1.83; df=19; P=0.08 in 2013; t-test=1.79; df=19; P=0.09 in 2014), but no oviposition ever occurred.

Only in 2014 both host location and oviposition was observed when the parasitoid was offered a non-target gall. In fact, 6 *T. sinensis* females out of the 20 tested showed interest in a non-target host, laying eggs in *A. curvator* galls (each female laid one egg per gall), although this was significantly lower that the number of *T. sinensis* females that subsequently oviposited on the control, *D. kuriphilus* galls (t-test=-3.25; df=19; P=0.004).

In the non-target galls tested no native parasitoid was detected by molecular analysis carried out after dissection. In the control trials oviposition occurred in 96% of the chestnut galls tested.

The cytochrome oxidase I gene obtained from each of the larvae found in the dissected galls, both on non-target and target hosts, was submitted to molecular identification, sequenced and the sequences compared with those in the National Centre for Biotechnology Information (NCBI) sequence database. In all cases, a minimum of 99% similarity with *T. sinenisis*-related sequences was observed.

<u>Olfactometer bioassays</u>

In the olfactometer bioassays all the *T. sinensis* females tested responded by making a choice within the fixed time. Higher numbers of *T. sinensis* females were attracted to the chestnut galls compared to non-target hosts. In particular, significant differences in the responses of adults were found when chestnut gall was compared to *A. cydoniae* (χ^2=6.53; df=1; P=0.01), and *A. grossulariae* (χ^2=13.33; df=1; P<0.01), while for *A. curvator* (χ^2=3.33; df=1; P=0.07), *A. multiplicatus* (χ^2=3.33; df=1; P=0.07), *B. pallida* (χ^2=3.33; df=1; P=0.07), and *N. quercusbaccarum* (χ^2=2.13; df=1; P=0.14) no significant differences were observed (Figure 2).

Discussion

In recent years there has been growing concern about the potential or actual threat presented by alien entomophagous biological control agents to populations of native non-target arthropod species (López-Vaamonde and Moore 1998). The use of tests to assess plants as potential hosts for herbivorous insects began over 70 years ago and has long been routine. In contrast, interest in estimating parasitoid and predator host ranges has lagged considerably behind (van Driesche and Murray 2004).

A full environmental risk assessment relies on the identification and evaluation of potential risks associated with natural enemy release and the development of a plan to minimize them. That is why in a classical biological program it is extremely important, prior to releasing the exotic natural enemy, to identify, assess and weigh all adverse and beneficial effects in a risk-cost benefit assessment (Gibbs et al. 2011).

The set of species that can support the development of a parasitoid or serve as prey for a predator—observed under laboratory conditions exclusively—is defined as the fundamental host range of a potential agent, also termed the physiological host range. In contrast, the ecological host range is defined as the current and evolving set of host species actually used for successful reproduction in the field (Onstad and Mcmanus 1996; Haye et al. 2005). However, assessment of the host range of a biological control agent in the laboratory often yields a significantly broader fundamental host range in comparison to the ecological host range (Haye et al. 2005), overestimating the field host range. Generally, the results of the host specificity study constitute a key factor in the risk analysis performed before an exotic beneficial arthropod can be safely utilized as a biological control agent.

In the last decade annual chestnut production in Italy underwent several drops, mainly ascribed to adverse climatic conditions and pests. In particular, the Asian chestnut gall wasp has been responsible for a severe reduction in fruiting, with yield losses estimated to reach between 65% and 85% in northern Italy (Bosio et al. 2013; Battisti et al. 2014), with a heavy economic impact on Italian chestnut production.

From the first report of this pest in Italy in 2002, following the successful experiences in Japan and North America, and due to the severity of the problem, pros and cons in the release of the exotic parasitoid were balanced, and biological control was considered the only economically and environmentally sustainable solution to deal with the pest promptly, since in the literature alternative approaches (e.g. chemical treatments, resistant varieties) were all found infeasible. In its native distribution, *D. kuriphilus* populations are controlled by natural enemies. In all the countries invaded by the pest a rich parasitoid community has been reported, but the attack rates have remained low (typically less than 2%) (Aebi et al., 2007; Gibbs et al., 2011; Quacchia et al., 2013).

Introductions of exotic organisms carry with it some unknown level of environmental risk, but these risks must be weighed against the consequences of not initiating biological control, which can also include serious environmental as well as economic consequences (Heimpel et al., 2004). The releases of *T. sinensis* carried out aided in restoring a habitat to similar conditions as those observed prior to the pest introduction, representing a large benefit for the chestnuts and chestnut growers.

Even though the host range of *T. sinensis* has never been studied or tested in detail in either its native or introduced ranges over a long period of time, the parasitoid was considered specific to *D. kuriphilus* (Murakami et al. 1977; Quacchia et al. 2014).

Nevertheless the host range of an apparently strictly monophagous parasitoid species may not be constant, either in space or time; it could expand in environments with greater diversity and hence have a larger number of new potential hosts (López-Vaamonde and Moore 1998). Attacking non-target hosts is of concern due to the potential harm that exotic natural enemies may impose on native or beneficial exotic species (Nadel et al. 2009). However, the risk to non-target species is complex and difficult to estimate; guidelines for appropriate host range tests have been proposed by the EFSA Panel on Plant Health (2010). Following this suggestion, our approach was to assess *T. sinensis*'s capacity to attack and reproduce on non-target gall species inhabiting common habitat with *Castanea* trees.

Even if, to date, no severe non-target effects have ever been reported in the literature after the release of *T. sinensis*, this paper represents the first report of potential negative effects on non-target native galls makers mainly present on oaks (*Quercus* spp.) by introducing the exotic parasitoid *T. sinensis* as a biological control agent for the chestnut gall wasp, *D. kuriphilus*. In contrast to Quacchia et al. (2008; 2014), who confirmed a high level of specificity for *T. sinensis* on the basis of a set of non-target species tested, our study highlights how *B. pallida* oak galls proved to be successfully parasitized. Even if the case record was low (3 galls parasitised by *T. sinensis* out of 856 collected in the field), this finding suggests that this oak gall species is a suitable host for the exotic parasitoid. In 2013 the emergence of *T. sinensis* was recorded only from *B. pallida* galls collected in both surveyed sites of Piemonte region; that is why, due to the considerable presence of *B. pallida* in our environment, a mass collection of this gall species was performed in 2014, but no other emergence was recorded. In the laboratory the conditions under which non-target tests are conducted may also limit interpretation of the host range. Test arenas confine the parasitoid with the host and may force encounters with non-target hosts, increasing the probability of the parasitoid accepting completely factitious hosts (Mason et al. 2011). Nevertheless, in the no choice oviposition trials, when they were offered to *T. sinensis* females oviposition only occurred in 2014 on *A. curvator*; since galls were dissected to detect the presence of the larvae, no data is available about their potential development to the adult stage. Some probing attempts were recorded on *B. pallida*, but no oviposition ever occurred.

And in general, *T. sinensis* proved to be more attracted by chestnut galls compared to non-target hosts, showing a similar responsiveness in the olfactometer bioassays as well. Statistical differences were observed only for *A. cydoniae* and *A. grossulariae*, but the interest showed by the parasitoid for *A. curvator*, *A. multiplicatus*, *B. pallida*, and *N. quercusbaccarum* have to be further investigated.

In the oviposition trials, all the parasitoid females were naïve and tests were conducted only under no choice conditions. In accordance with Withers and Brown (2005), no choice tests with both naïve and oviposition-experienced females should be performed because it has been shown that oviposition experience may either reduce or enhance responsiveness. Furthermore, since parasitoids can display wider host ranges in choice tests, it would be useful to set up choice trials where the parasitoid is given a choice of host and non-target host for a more accurate prediction of potential host range. In fact, parasitoid response is generally expected to be biased toward the familiar host, especially after the parasitoid successfully oviposits in it (Nadel et al. 2009).

In this study we confirmed that *T. sinensis* has a broader physiological host range than reported in the literature and determined that it may be attracted by non-target hosts other than *D. kuriphilus*. The assessment of risk requires consideration of the likelihood and magnitude of an effect and evaluation of risk management priorities (Moeed et al., 2006). Methods for quantifying the magnitude and spatiotemporal scale of impact of exotic natural enemies on populations of native insects are crucial to advance current risk assessment (Wyckhuys et al., 2009), however, the incidence of these host shift in the complex chestnut-oak is currently difficult to be quantified in the natural environments.

Over the 2-year period, in order to monitor the potential emergence of *T. sinensis*, 8 oak gall species suggested in the EFSA list were collected, while in the oviposition trials 4 non-target species out of 9 were tested. At present, research is ongoing to test *A. cydoniae*, *A. inflator*, *A. lucidus*, *A. multiplicatus*, and *D. cerriphilus* in controlled conditions as possible hosts for *T. sinensis*, since they provide the closest phenological match to the flight period of the parasitoid (i.e. between April and May in Italy) (EFSA Panel On Plant Health Aebi et al., 2011). Further investigations need also to be performed on *B. pallida*, given the emergence of the parasitoid from galls collected in the field and the interest showed by some females in the oviposition trials.

Food availability is an important aspect that may influence the biological traits of many arthropods. Recently a novel insight concerning *T. sinensis'*s life cycle was highlighted in this regard: a prolongation of diapause was in fact reported. Even if it is hard to speculate what are the factors that triggered this response, this may be read as an adaptive value to protect the population against the yearly fluctuation in food supply (Ferracini et al. 2015). At present, there is growing evidence that the *T. sinensis* parasitism rate is dramatically increasing in some Italian regions, almost reaching 98% in old release sites (Bosio et al., 2013), and parasitisation on non-target hosts was recorded only in sites where a stable population of the exotic parasitoid is present, (Piemonte region), since the first releases date back to 2005. Hence, a host-shift to oak galls may be due to the need to find another suitable host since populations of the Asian chestnut gall wasp have recently declined significantly (Ferracini et al. 2015), and even though the frequency of cases of observed non-target impacts were small, major effects on non-target galls populations could be expected to be detectable. Hence, longer term studies are necessary to allow more precise conclusions to be drawn on non target impacts, that is why an exhaustive research about all potential non-target galls and their phenology is needed in order to better understand the relationship between the exotic parasitoid and native biocoenoses. At the same time, since in this paper four native *Torymus* species emerged from non target galls during *T. sinensis* flight period as well, and in literature five native species [*T. auratus*, *T. erucarum* (Schrank), *T. flavipes*, *T. geranii*, and *T. scutellaris* (Walker)] are reported from chestnut galls (Alma et al., 2015), an evaluation of the potential for hybridization between these congeneric species is also required in order to have a comprehensive knowledge of the environmental risk to non-target species that *T. sinensis* may pose to native biodiversity.

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Table 1 Years of release and coordinates of *T. sinensis* release points in the surveyed sites

Italian regions	Surveyed sites	Year of release	Coordinates
Liguria	Borzonasca	2012	44°26'01.6"N 9°23'45.9"E
	Sassello	2011	44°29'33.7"N 8°33'17.9"E
Piemonte	Molare	2012	44°34'40.2"N 8°36'10.5"E
	Pianfei	2008	44°19'41.2"N 7°40'58.9"E
Toscana	Marradi	2010	44°04'53.1"N 11°38'17.8"E
	Piazza al Serchio	2011	44°10'31.8"N 10°17'14.9"E
Valle d'Aosta	Arnad	2012	45°38'22.7"N 7°43'41.8"E
	Perloz	2012	45°36'53.9"N 7°48'24.4"E

Table 2 Number of non-target galls collected in the two-year period (2013–2014) from the surveyed sites according to the species list for host-specificity testing established by EFSA Panel On Plant Health (2010).

Year	**Italian regions**	**Host plant**	**Galls species**	**Generation**	**No.**
2013	Liguria	*Quercus robur*	*Andricus curvator*	sexual	1
		Quercus petraea	*Biorhiza pallida*	sexual	29
	Piemonte	*Quercus robur*	*Andricus curvator*	sexual	26
		Quercus cerris	*Andricus cydoniae*	sexual	205
		Quercus robur	*Andricus inflator*	sexual	46
		Quercus cerris	*Andricus multiplicatus*	sexual	12
		Quercus robur	*Biorhiza pallida*	sexual	132
		Rosa canina	*Diplolepis rosae*	asexual	17
		Quercus robur	*Neuroterus quercusbaccarum*	sexual	2
	Toscana	*Quercus cerris*	*Andricus lucidus*	sexual	1
		Quercus cerris	*Andricus multiplicatus*	sexual	41
		Rosa spp.	*Diplolepis rosae*	asexual	1
	Valle d'Aosta	*Quercus robur*	*Andricus curvator*	sexual	61
		Quercus cerris	*Andricus cydoniae*	sexual	2
		Quercus cerris	*Andricus grossulariae*	sexual	3
		Quercus pubescens	*Andricus inflator*	sexual	12
		Quercus pubescens	*Biorhiza pallida*	sexual	317
		Rosa canina	*Diplolepis rosae*	asexual	6
		Quercus pubescens	*Neuroterus quercusbaccarum*	sexual	79
2014	Liguria	*Quercus robur*	*Biorhiza pallida*	sexual	76
	Piemonte	*Quercus robur*	*Biorhiza pallida*	sexual	150
	Toscana	*Quercus petraea*	*Biorhiza pallida*	sexual	10
	Valle d'Aosta	*Quercus robur*	*Biorhiza pallida*	sexual	142

Table 3 Numbers of native torymid species and the exotic *Torymus sinensis* (in bold) emerged from non-target galls collected in the 2-year period (2013–2014) from the surveyed sites.

Year	**Italian regions**	**Gall species**	**Torymid species emerged**	♀♀	♂♂
2013	Liguria	*Biorhiza pallida*	*Torymus affinis*	9	6
			Torymus auratus	40	36
			Torymus flavipes	20	3
	Piemonte	*Andricus cydoniae*	*Megastigmus dorsalis*	0	1
		Biorhiza pallida	*Torymus affinis*	10	13
			Torymus auratus	0	2
			Torymus geranii	0	1
			Torymus sinensis	0	3
		Diplolepis rosae	*Torymus auratus*	1	3
	Toscana	*Andricus lucidus*	*Megastigmus dorsalis*	4	0
		Andricus multiplicatus	*Megastigmus dorsalis*	0	1
	Valle d'Aosta	*Andricus curvator*	*Torymus flavipes*	27	44
		Andricus cydoniae	*Torymus flavipes*	1	0
		Biorhiza pallida	*Torymus affinis*	3	7
			Torymus auratus	11	14
			Torymus flavipes	105	105
			Torymus geranii	0	2
		Neuroterus quercusbaccarum	*Torymus flavipes*	24	50
2014	Liguria	*Biorhiza pallida*	*Torymus auratus*	4	8
	Piemonte	*Biorhiza pallida*	*Torymus affinis*	23	40
			Torymus auratus	2	0
	Toscana	*Biorhiza pallida*	*Torymus affinis*	7	4
			Torymus auratus	3	1
	Valle d'Aosta	*Biorhiza pallida*	*Torymus affinis*	84	69
			Torymus auratus	2	2
			Torymus flavipes	0	2

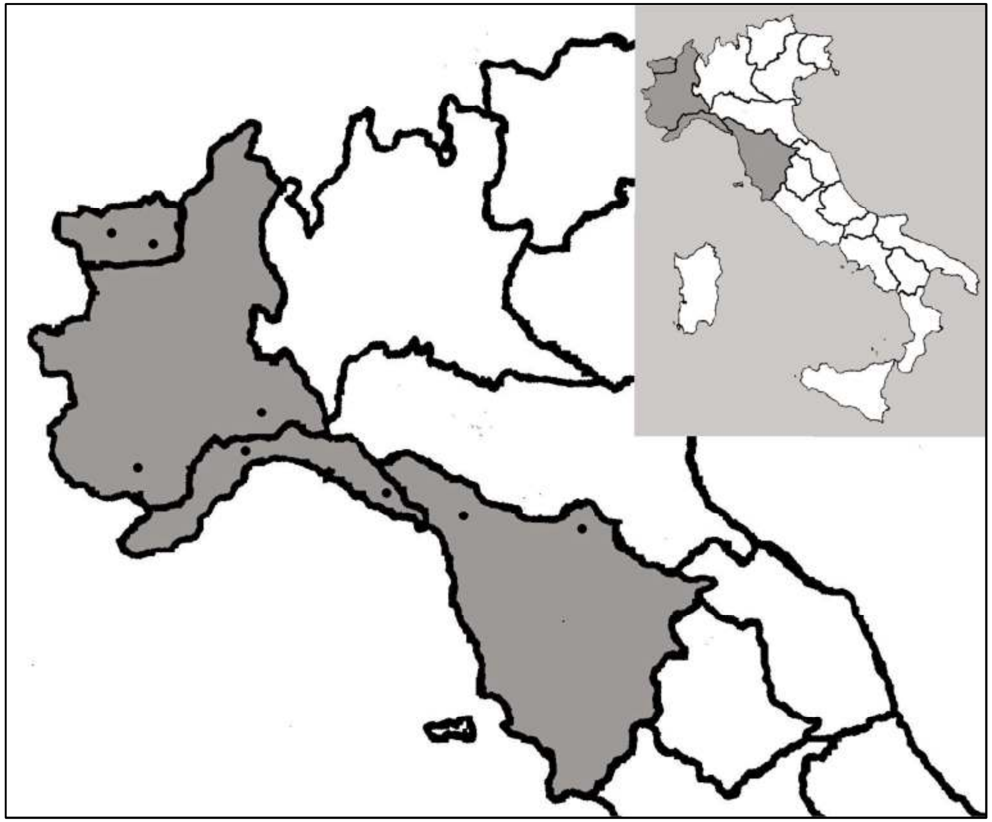
Table 4 Mean number (±SE) of host location, attempted oviposition, and oviposition events engaged in by *T. sinensis* females comparing non-target oak galls[+] and *D. kuriphilus* galls (control) recorded during 1 h observation periods in no choice oviposition trials over a two-year period (2013–2014). Means were compared for each non-target species using a paired t-tests for dependent samples; P<0.05 *; P<0.01 **; P<0.001***.

Year	Non-target species compared to the control	No.	Host location	Attempted oviposition	Oviposition
2013	*Andricus curvator*	30	0.33± 0.09***	0.00	0.00***
	Dryocosmus kuriphilus	30	0.97± 0.10	0.00	0.83± 0.07
	Biorhiza pallida	20	1.40± 0.29	0.15± 0.08	0.00***
	Dryocosmus kuriphilus	20	1.45± 0.15	0.00	0.80± 0.09
	Andricus grossulariae	20	0.85± 0.15**	0.00	0.00***
	Dryocosmus kuriphilus	20	1.55± 0.17	0.00	0.90± 0.07
	Neuroterus quercusbaccarum	20	0.80± 0.12***	0.00	0.00***
	Dryocosmus kuriphilus	20	1.90± 0.22	0.00	0.80± 0.09
2014	*Andricus curvator*	20	0.35± 0.11**	0.00	0.30 ± 0.11**
	Dryocosmus kuriphilus	20	0.95± 0.15	0.00	0.80± 0.09
	Biorhiza pallida	20	1.50± 0.44	0.25± 0.14	0.00***
	Dryocosmus kuriphilus	20	1.55± 0.18	0.00	0.90± 0.07

[+] The selection was based on the species list for host-specificity testing established by EFSA Panel On Plant Health (2010)

Figure 1 Location of the sampling sites (black dots). The inset indicates the location of the four surveyed regions in Italy.

Figure 2 Responses of *T. sinensis* (number of responding females in bars) in a Y-tube olfactometer to the odours of chestnut gall and non-target galls. Numbers in bars represent individuals that moved toward the volatiles. Chi-square statistics (* P <0.05; df=1) tested the hypothesis that the distribution of side arm choices deviated from a null model where odour sources were chosen with equal frequency.



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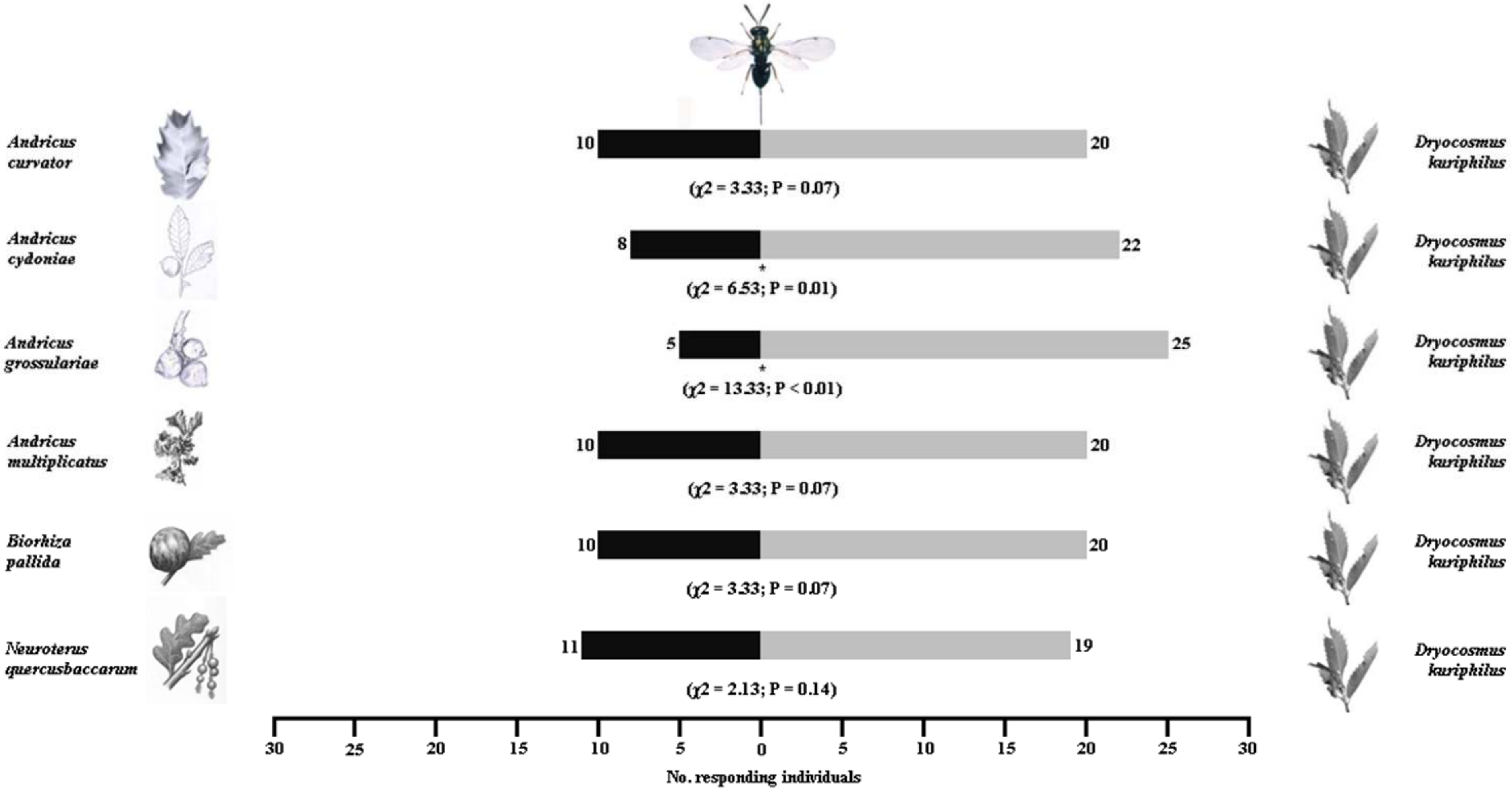
Andricus curvator
10
20
(χ2 = 3.33; P = 0.07)
Dryocosmus kuriphilus
Andricus cydoniae
8
22
*
(χ2 = 6.53; P = 0.01)
Dryocosmus kuriphilus
Andricus grossulariae
5
25
*
(χ2 = 13.33; P < 0.01)
Dryocosmus kuriphilus
Andricus multiplicatus
10
20
(χ2 = 3.33; P = 0.07)
Dryocosmus kuriphilus
Biorhiza pallida
10
20
(χ2 = 3.33; P = 0.07)
Dryocosmus kuriphilus
Neuroterus quercusbaccarum
11
19
(χ2 = 2.13; P = 0.14)
Dryocosmus kuriphilus
30 25 20 15 10 5 0 5 10 15 20 25 30
No. responding individuals