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Post-release evaluation of non-target effects of *Torymus sinensis*, the biological control agent of *Dryocosmus kuriphilus* in Italy

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Abstract A post-release study was performed to assess the impact of *Torymus sinensis* (Hymenoptera: Torymidae), the biological control agent of *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae), on native cynipid gall inducers in Italy. In total, 14,512 non-target galls were collected, corresponding to seven genera: *Andricus*, *Aphelonyx*, *Biorhiza*, *Cynips*, *Diplolepis*, *Neuroterus*, and *Synophrus*, and 8708 chalcid parasitoids were recorded. The Torymidae family accounted for about 30%, and *Bootanomyia* (=*Megastigmus*) *dorsalis*, *Torymus affinis* and *T. flavipes* were the most represented species. A total of 116 *T. sinensis* emerged from 15 different oak galls, mainly *Andricus curvator* and *A. inflator*. In controlled conditions, oviposition was recorded on *A. cydoniae*, *A. grossulariae* and *A. lucidus*, while no mating with native congeneric species occurred. This paper confirms the realised host-range expansion by *T. sinensis*. Even if it were extremely difficult to evaluate its magnitude, the impact appears minimal, and an occasional feeding with no changes in the distribution or abundance of non-target hosts is expected.

Keywords *Torymus sinensis* · Native chalcid parasitoids · Non-target effects · Environmental risk assessment · Torymidae · Cynipid gall inducers

Introduction

Alien species are recognised as the second largest threat to biological diversity, the first being habitat destruction. Exotic pests, in the absence of their natural antagonists, may cause unprecedented damage to native biodiversity and the economic impact of invasive pests can be great. The importance of natural enemies for pest control has been known for over a thousand years, reaching all-time highs in the 1960s and 1970s (Hajek et al. 2016). In this context, classical biological control (CBC), the importation and release of an organism outside its natural range to control a pest, attempts to manage invasive pests through the introduction of exotic natural enemies. The release of more than 2000 species of natural enemies has resulted in the permanent reduction of at least 165 pest species worldwide (Cock et al. 2010; Hajek et al. 2016). The most striking benefit, when compared with any pest control program based on pesticides, is that they can be permanent and self-propagating, and moreover the

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risks of pesticide resistance are avoided (Boettner et al. 2000; De Clercq et al. 2011; Naranjo et al. 2015; van Driesche et al. 2010). Many examples of successful CBC can be listed worldwide: the vedalia beetle *Rodolia cardinalis* Mulsant (Coleoptera: Coccinellidae) against the cottony cushion scale *Icerya purchasi* Maskell (Hemiptera: Margarodidae) (De Clercq et al. 2011); the egg parasitoid *Anaphes nitens* (Hymenoptera: Mymaridae) against the Australian weevil *Gonipterus scutellatus* (Coleoptera: Curculionidae) (Hanks et al. 2000) in California, (USA); the parasitoid *Epidinocarpis lopezi* De Santis (Hymenoptera: Encyrtidae) for the control of the cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidea) in Africa (Chakupurakal et al. 1994) and the wasp *Neodryinus typhlocybae* (Ashmead) (Hymenoptera: Dryinidae) to control the flatid planthopper *Metcalfa pruinosa* (Say) (Homoptera: Flatidae) in North America and Europe (Alma et al. 2005).

Nevertheless, the irreversible introduction of a biological control agent (BCA) might bring, with time, negative effects, either direct or indirect, in particular on native non-target species. The outcomes may range on a large scale from negligible to massive effects, the latters especially on vertebrates or molluscs, and are difficult to predict in complex systems (De Clercq et al. 2011; Louda et al. 2003). Concerns about the safety of CBC and its possible consequences have been rising, in particular about their non-transient effects on the environment, such as impacts on natural biodiversity, host switching and dispersal into non-agricultural habitats (De Clercq et al. 2011; Louda et al. 2003; Thomas and Willis 1998).

Even if reports of significant environmental impacts are increasing (Boettner et al. 2000; Funasaki et al. 1988; Howarth 1991; Louda et al. 2003), and some early CBC programs, especially concerning introduction to islands, have had severe consequences for non-target organisms (Lynch et al. 2001), Hajek et al. (2016) underline how very few cases of quantified negative ecological effects on native species or ecosystem have been documented, or in other cases suspected but not verified, and how no large-scale extinction has yet been reported (Suckling and Sforza 2014). One of the most successful examples of recent European CBC programs is surely represented by the introduction of the Chinese parasitoid *Torymus sinensis* Kamijo (Hymenoptera: Torymidae), to control the Asian chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) (ACGW). This parasitoid was first released in Italy in 2005, and following the positive Italian experience further release programs were performed in Croatia, France and Hungary, as well as test releases in Spain and Portugal (Ferracini and Alma 2015; Ferracini et al. 2015a; Matošević et al. 2014; Paparella et al. 2016).

The urgent need for a full environmental risk assessment and the increasing concern about CBC and its consequences on natural biodiversity, made necessary the evaluation of the possible adoption by *T. sinensis* of alternative native hosts. Furthermore, another potential environmental impact of biological control is represented by hybridisation between introduced BCA's and native species. The evidence of hybridisation reported by Yara et al. (2010) between the introduced *T. sinensis* and the native *Torymus* species (i.e. *T. beneficus*) in Japan, highlights the need for knowledge regarding potential adverse consequences towards the native congeneric species.

Since the EFSA Panel on Plant Health selected a non-target species list for testing the host-specificity of *T. sinensis* on the basis of their closest phenological match to the flight period of the parasitoid (i.e. between April and May in Italy) (EFSA Panel on Plant Health (PLH) 2010), previous investigations about the host-range of *T. sinensis* were performed in NW Italy. Four non-target oak galls [*Andricus curvator* Hartig, *A. grossulariae* Giraud, *Biorhiza pallida* Olivier and *Neuroterus quercusbaccarum* (L.)] were tested in no-choice oviposition trials and olfactometer bioassays in controlled conditions, showing the adoption by the exotic parasitoid of the non-target *B. pallida* galls (Ferracini et al. 2015a).

On the basis of this recent evidence and with the aim to carry out further research on a broader scale, an exhaustive post-release study was performed. Investigations were carried out in a three-year period (2013–2015) in North-central Italy where *T. sinensis* is established, in order to study the native parasitoid complex associated with non-target hosts, in particular native cynipid species inducing galls on oaks and wild rose. Hence, non-target galls were collected to evaluate the possible adaptation of this exotic wasp as well. Moreover, *T. sinensis* adults were exposed to non-target hosts and native *Torymus* species in no-choice conditions to perform oviposition and hybridisation trials, respectively.

Materials and methods

Gall collection

In order to evaluate the potential host-range expansion of *T. sinensis* from chestnut to non-target hosts, collection was carried out in 86 sites in nine Italian regions (Abruzzo, Aosta Valley, Emilia Romagna, Latium, Liguria, Lombardy, Piedmont, Tuscany, and Trentino Alto Adige) in the three-year period 2013-2015. Samplings were performed 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.). Sampled trees and shrubs were located in mixed forests close to chestnut stands, where a stable *T. sinensis* population had been previously assessed. Additional data about the years of sampling, the regions and the coordinates of the sites are provided in supplementary Table S1. Collection, isolation and maintenance of the galls were performed according to the method described by Ferracini et al. (2015a).

Unparasitised fresh galls from chestnut trees (used as a control) were collected in the Trentino Alto Adige region in a site with no presence of *T. sinensis,* to perform oviposition trials. Parasitised withered chestnut galls were collected once a year, in winter, in the Piedmont region (Italy), in chestnut orchards where the parasitoid was first released in 2005 and then successfully established, to obtain *T. sinensis* adults to be used in the hybridisation trials.

Insect

Native *Torymus* spp. used in the hybridisation trials emerging from non-target galls, and *T. sinensis* specimens emerging from chestnut galls were kept individually in glass tubes, closed with a cotton plug, with drops of honey on cardboard, and kept in a climatic chamber at 15 ± 1 °C, 60 ± 5% RH, and a photoperiod of 16:8 (L:D) h, until the trials. The other specimens were stored in 99% alcohol after their emergence.

Hybridisation trials

Only naïve, six-day-old, unmated individuals were used for the trials for both native parasitoids and *T. sinensis*. Courtship and mating behaviour between *T. sinensis* and five native *Torymus* species emerged from oak galls were evaluated. In particular, *T. auratus* Muller (11 males and 19 females), *T. affinis* Fonscolombe (six males and nine females), *T. flavipes* Walker (ten males and four females), *T. cyaneus* Walker (four males and four females) and *T. geranii* Walker (one male and one female) were used.

Behavioural events were named according to preliminary observations performed (Table 1). All observations took place in an arena consisting of a Petri dish (50 mm diameter) with a filter paper sheet. The number and duration of all the behavioural events were recorded for 45 min using JWatcher® software. A native *Torymus* male parasitoid was placed inside the arena together with a contemporary female of *T. sinensis.* At the end of the trials, the experienced *T. sinensis* female was transferred into another arena, with a naïve conspecific of the opposite sex used as control, to verify if mating occurred. Similarly, a native *Torymus* female was tested together with a male *T. sinensis*, which, at the end of the trial, was transferred in another arena with a conspecific individual to verify if mating occurred, as control. At the end of the trials, the native parasitoid was stored in 99% alcohol for morphological and/or molecular identification.

No-choice oviposition trials

Mated six-day-old naïve females were used. One day before the trials, the female was placed in a plastic tube at room temperature together with three males to ensure mating. Eight galls out of the nine included in the oak host gall species list for host-specificity testing established by the EFSA Panel on Plant Health (PLH) (2010) (*A. curvator*, *A. cydoniae* Giraud, *A. grossulariae*, *A. inflator* Hartig, *A. lucidus* Hartig, *A. multiplicatus* Giraud, *B. pallida*, and *N. quercusbaccarum*) were tested, in addition to *Diplolepis rosae* L. All the non-target gall species belonged to the sexual generation, except for *D. rosae* (asexual generation), and *A. lucidus* (both sexual and asexual generations).

Table 1 Behavioural events recorded during the hybridisation trials exposing a native *Torymus* parasitoid to a *T. sinensis* of the opposite sex

Behaviour	Description
Ignoring	Both individuals show no interest in the partner
Courtship dance	The male searches for the female, swinging and flapping his wings, more and more rapidly as he approaches the female
Contact with the antennae	The male mounts the female and places his antennae between those of the female, making repeated contact with her antennae
Mating	The receptive female stays still, lifting her abdomen up, while the male moves backwards and curved his abdomen downwards. Copula terminates when the female starts walking away and the male dismounted
Attempted mating	The male mounts the female, tries or succeeds in making contact with her antennae, but the female either pushes the male away with her hind legs or continues walking or flats her abdomen on the floor, preventing the copulatory act

A single fresh non-target gall was offered to a *T. sinensis* female placed on a filter paper sheet inside a Petri dish (diameter 10 cm). For each gall species 15 replications were performed, except for *D. rosae* for which only ten galls were found. Three behavioural sequences were recorded, as described in Table 2. The time spent for gall detection was calculated as the time elapsed from the female's entrance into the arena and the contact with the tested gall. Observations were performed under a stereomicroscope for 30 min, using JWatcher® software. The average duration of each recorded behavior was compared with the one recorded on *D. kuriphilus* galls. At the end of the trial, the female was left in the Petri dish with the gall for an additional 24 h and then removed. Since eggs might have escaped detection, galls were then stored in a climatic chamber at 24 ± 2 °C, 50 ± 10% RH, and a photoperiod of 16:8 (L:D) h for ten days to ease the detection of the parasitoid at larval stage. All the trials were performed under laboratory conditions. To avoid any influence on the behaviour of the parasitoid, chestnut galls were collected in the Trentino 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.

Parasitoid identification

All the parasitoids emerged from non-target hosts were morphologically identified using specific dichotomous keys (Alkhatib et al. 2014; Askew 1961a, b; Askew and Nieves-Aldrey 2000; de Vere Graham and Gijswijt 1998; Kamijo 1982; Nieves-Aldrey and Askew 1988; Roques and Skrzypczyńska 2003) and by comparison with voucher specimens deposited at the DISAFA-Entomology laboratory, Grugliasco, Italy. 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). Parasitoids

Table 2 Behavioural events recorded during the no-choice oviposition trials when single fresh non-target galls were individually offered to a *T. sinensis* female

Behaviour	Description
Gall detection	Time elapsed between the entrance of the female inside the arena and the first contact with the gall
Drumming	The female walks on the gall with her antennae touching the gall and drumming for host location
Probing	The female repeatedly inserts the ovipositor for few seconds and retracts it quickly
Oviposition	The female inserts the ovipositor and lays her eggs, with a typical pumping movement of the abdomen. Oviposition was considered successful only if it lasted more than 60 s

developed upon inquilines or other insects, such as aculeate Hymenoptera, Coleoptera and Neuroptera, were discarded.

Statistical analysis

In the hybridisation trials, the time of duration of the following behaviours was recorded: courtship dance, antennal contact, attempted mating and mating. The average time of each behaviour was compared with those recorded in the control trials by non-parametric Wilcoxon signed-rank test ($P < 0.05$). In the no-choice oviposition trials the times that *T. sinensis* females spent in four types of behaviour (gall identification, antennal drumming, probing and oviposition) were recorded, and averages were analysed for each non-target gall and compared with those recorded on ACGW galls (as control) by non-parametric Mann–Whitney *U* test ($P < 0.05$). All analyses were performed using SPSS version 22.0 (SPSS, Chicago, IL, USA).

Results

In total, 14,512 non-target galls were collected, corresponding to seven different genera: *Andricus*, *Aphelonyx*, *Biorhiza*, *Cynips*, *Diplolepis*, *Neuroterus*, and *Synophrus* (Table 3). The galls found most frequently were the sexual generation of *B. pallida* (1886), and the asexual generations of *Andricus quercustozae* Bosc (1106) and *A. lignicolus* Hartig (1049).

The number of chalcid parasitoids emerged from the non-target galls is reported in supplementary Table S2. In total 8708 parasitoids from the superfamily Chalcidoidea emerged, and thirty-five species were identified using morphological characters and molecular analyses, distributed over six chalcid families (Eulophidae, Eupelmidae, Eurytomidae, Pteromalidae, Torymidae and Ormyridae). The most frequent species were *Aulogymnus skianeuros* Ratzeburg (964), *Bootanomyia* (=*Megastigmus*) *dorsalis* F. (1054), and *Sycophila biguttata* Swederus (787). A total of 2668 native torymid specimens emerged, belonging to three genera: *Bootanomyia* (=*Megastigmus*), *Glyphomerus* and *Torymus*.

A total of 116 *T. sinensis* was recorded as well, representing 1.3% of the total number of parasitoids emerged. The non-target galls involved were *A. caputmedusae* Hartig (8), *A. coronatus* Giraud (1), *A. curvator* (35), *A. cydoniae* (4), *A. dentimitratus* Rejtõ (1), *A. inflator* (29), *A. kollari* Hartig (3), *A. lignicolus* (1), *A. lucidus* (3), *A. quercustozae* (16), *B. pallida* (9), *Cynips quercusfolii* L. (2), *N. anthracinus* Curtis (2), *N. quercusbaccarum* (1), and *Synophrus politus* Hartig (1).

The parasitism by the introduced agent calculated for each non-target gall ranged between 0.1 and 1.6% for all the non-target galls collected, except for *A. curvator* (3.5%) and *A. inflator* (5.7%). In particular, for these two last non-target gall species the relative dominance by *T. sinensis* considering the whole parasitoid complex emerged was of 21.7 and 49.2%, respectively.

Hybridisation trials

When native individuals were exposed to *T. sinensis*, only a courtship dance was observed, as shown in Fig. 1, while no contact with the antennae, attempted mating or mating behaviour was ever recorded. When male native parasitoids were exposed to a *T. sinensis* female, the average duration of a single dancing event was always significantly lower compared to the control (Wilcoxon signed-rank test; *T. auratus*: $Z = -2.134$, $P = 0.033$; *T. affinis*: $Z = -2.201$, $P = 0.028$; *T. flavipes*: $Z = -42.803$, $P = 0.005$), except for *T. cyaneus* (Wilcoxon signed-rank test; $Z = -1.826$, $P = 0.068$).

When a female native parasitoid was tested together with a *T. sinensis* male, the average duration of a single dancing event was significantly lower compared to the control for *T. auratus* (Wilcoxon signed-rank test; $Z = -3.724$, $P < 0.001$), while no differences were recorded for *T. affinis, T. flavipes* and *T. cyaneus* (Wilcoxon signed-rank test; *T. affinis*: $Z = -1.955$, $P = 0.051$; *T. flavipes*: $Z = -1.826$, $P = 0.068$; *T. cyaneus*: $Z = 0$, $P = 1.000$). No courtship was recorded for *T. geranii* either, but no statistical analysis was performed, due to the low number of individuals available. All control trials using *T. sinensis* individuals resulted in successful mating.

No-choice oviposition trials

The average times spent during the entire trial for gall identification, antennal drumming, probing and oviposition are reported in Fig. 2. The time needed for gall

Table 3 Number of the non-target galls collected in the three-year period (2013–2015) in the surveyed sites

Gall species	Generation	Gall structure	Year			Total
			2013	2014	2015	
Andricus amblycerus	Asexual	Unilocular	7	0	43	50
Andricus aries	Asexual	Unilocular	0	0	124	124
Andricus caliciformis	Asexual	Unilocular	18	0	30	48
Andricus caputmedusae	Asexual	Unilocular	10	166	332	508
Andricus conglomeratus	Asexual	Unilocular	0	0	89	89
Andricus coriarius	Asexual	Multilocular	0	15	103	118
Andricus coronatus	Asexual	Unilocular	0	4	104	108
Andricus curvator	Sexual	Unilocular	291	0	713	1004
Andricus cydoniae	Sexual	Multilocular	173	0	198	371
Andricus dentimitratus	Asexual	Unilocular	0	0	64	64
Andricus foecundatrix	Asexual	Unilocular	82	70	155	307
Andricus galeatus	Asexual	Unilocular	0	2	73	75
Andricus gallaeurnaeformis	Asexual	Unilocular	2	0	20	22
Andricus glutinosus	Asexual	Unilocular	8	95	51	154
Andricus grossulariae	Asexual	Multilocular	0	0	16	16
	Sexual	Unilocular	23	0	79	102
Andricus infectorius	Asexual	Unilocular	0	55	0	55
Andricus inflator	Asexual	Unilocular	5	0	12	17
	Sexual	Unilocular	61	1	445	507
Andricus kollari	Asexual	Unilocular	195	36	476	707
Andricus lignicolus	Asexual	Unilocular	226	172	651	1049
Andricus lucidus	Asexual	Multilocular	17	51	886	954
	Sexual	Multilocular	1	0	7	8
Andricus mitratus	Asexual	Unilocular	0	8	2	10
Andricus multiplicatus	Sexual	Multilocular	39	31	318	388
Andricus polycerus	Asexual	Unilocular	154	21	265	440
Andricus quercustozae	Asexual	Unilocular	34	296	776	1106
Andricus sieboldi	Asexual	Unilocular	0	0	28	28
Andricus solitarius	Asexual	Unilocular	121	0	44	165
Andricus sternlichti	Asexual	Unilocular	5	80	228	313
Aphelonyx cerricola	Asexual	Unilocular	139	25	129	293
Biorhiza pallida	Sexual	Multilocular	1470	61	355	1886
Cynips cornifex	Asexual	Unilocular	137	3	50	190
Cynips disticha	Asexual	Unilocular	0	3	42	45
Cynips divisa	Asexual	Unilocular	229	10	11	250
Cynips quercusfolii	Asexual	Unilocular	191	91	392	674
Diplolepis rosae	Asexual	Multilocular	10	6	61	77
Neuroterus albipes	Asexual	Unilocular	12	0	0	12
Neuroterus anthracinus	Asexual	Unilocular	85	102	375	562
Neuroterus lanuginosus	Asexual	Unilocular	50	3	73	126
Neuroterus minutulus	Asexual	Unilocular	18	0	64	82
Neuroterus quercusbaccarum	Asexual	Unilocular	143	29	409	581
	Sexual	Unilocular	169	8	131	308
Synophrus politus	Sexual	Unilocular	236	10	273	519
Total			4361	1454	8697	14,512

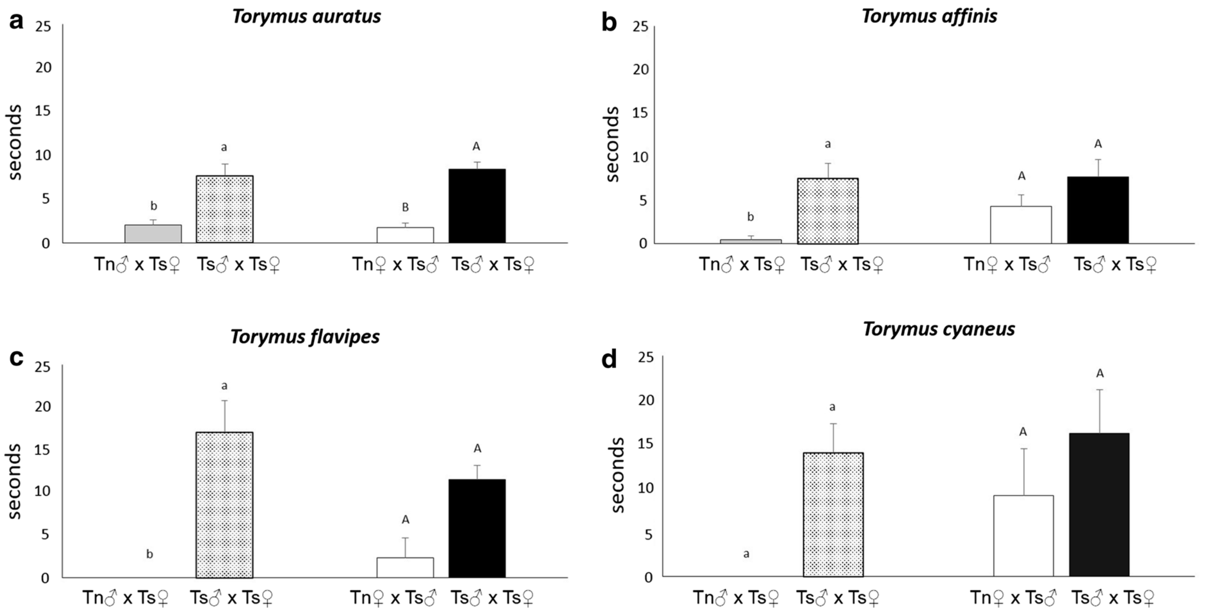

Fig. 1 Average duration (+ SE) of courtship dance events. Interspecific trials were performed exposing four native *Torymus* parasitoids (Tn) [*T. auratus* (**a**), *T. affinis* (**b**), *T. flavipes* (**c**), and *T. cyaneus* (**d**)] to *T. sinensis* (Ts) specimens of the opposite sex. Interspecific trials using male native parasitoids (Tn♂ × Ts♀) are indicated in grey and compared with their control trials (Ts♂ × Ts♀) indicated in dotted bars. Bars are topped with lowercase letters and the same letter represents averages that are not statistically different (Wilcoxon signed-rank test, $P < 0.05$). Interspecific trials using female native parasitoids (Tn♀ × Ts♂) are indicated in white and compared with their control trials (Ts♂ × Ts♀) indicated in black. Bars are topped with uppercase letters and the same letter represents averages that are not statistically different (Wilcoxon signed-rank test, $P < 0.05$)

identification was significantly higher when a *T. sinensis* female was offered single fresh non-target galls, compared to ACGW galls, used as control (Mann–Whitney *U* test; *A. curvator*: $Z = -3.915$; $P < 0.001$; *A. cydoniae*: $Z = -3.756$; $P < 0.001$; *A. grossulariae*: $Z = -3.758$; $P < 0.001$; *A. inflator*: $Z = -4.070$; $P < 0.001$; *A. lucidus* asexual generation: $Z = -3.332$; $P < 0.001$; *A. lucidus* sexual generation: $Z = -2.615$; $P = 0.008$; *A. multiplicatus*: $Z = -3.715$; $P < 0.001$; *B. pallida*: $Z = -3.655$; $P < 0.001$; *N. quercusbaccarum*: $Z = -4.143$; $P < 0.001$; *D. rosae*: $Z = -3.631$; $P < 0.001$). The time spent for antennal drumming on non-target galls was always significantly lower compared to the control (Mann–Whitney *U* test; *A. curvator*: $Z = -4.015$; $P < 0.001$; *A. cydoniae*: $Z = -2.473$; $P = 0.013$; *A. grossulariae*: $Z = -3.015$; $P = 0.002$; *A. inflator*: $Z = -3.636$; $P < 0.001$; *A. lucidus* asexual generation: $Z = -2.586$; $P = 0.010$; *A. lucidus* sexual generation: $Z = -4.478$; $P < 0.001$; *A. multiplicatus*: $Z = -2.249$; $P = 0.023$; *B. pallida*: $Z = -4.232$; $P < 0.001$; *N. quercusbaccarum*: $Z = -4.550$; $P < 0.001$; *D. rosae*: $Z = -4.038$; $P < 0.001$). The time spent in the probing activity was significantly lower only for *A. curvator*, *A. inflator*, *A. lucidus* sexual generation, *B. pallida*, N. *quercusbaccarum* and *D. rosae* (Mann–Whitney *U* test; *A. curvator*: $Z = -2.185$; $P = 0.037$; *A. inflator*: $Z = -2.769$; $P = 0.010$; *A. lucidus* sexual generation: $Z = -3.629$; $P = 0.001$; *B. pallida*: $Z = -3.324$; $P = 0.002$; *N. quercusbaccarum*: $Z = -3.507$; $P = 0.001$; *D. rosae*: $Z = -3.227$; $P = 0.002$), while no differences were measured for the other non-target galls compared to the control (Mann–Whitney *U* test; *A. cydoniae*: $Z = -0.649$; $P = 0.539$; *A. grossulariae*: $Z = -0.829$; $P = 0.436$; *A. lucidus* asexual generation: $Z = -1.050$; $P = 0.325$; *A. multiplicatus*: $Z = -0.617$; $P = 0.567$).

Oviposition was considered successful when the female spent more than 60 s with the ovipositor inserted in the gall (authors' observation). Oviposition was recorded on three non-target gall species: *A. cydoniae*, *A. grossulariae* and *A. lucidus* asexual generation. The total time spent for the oviposition on

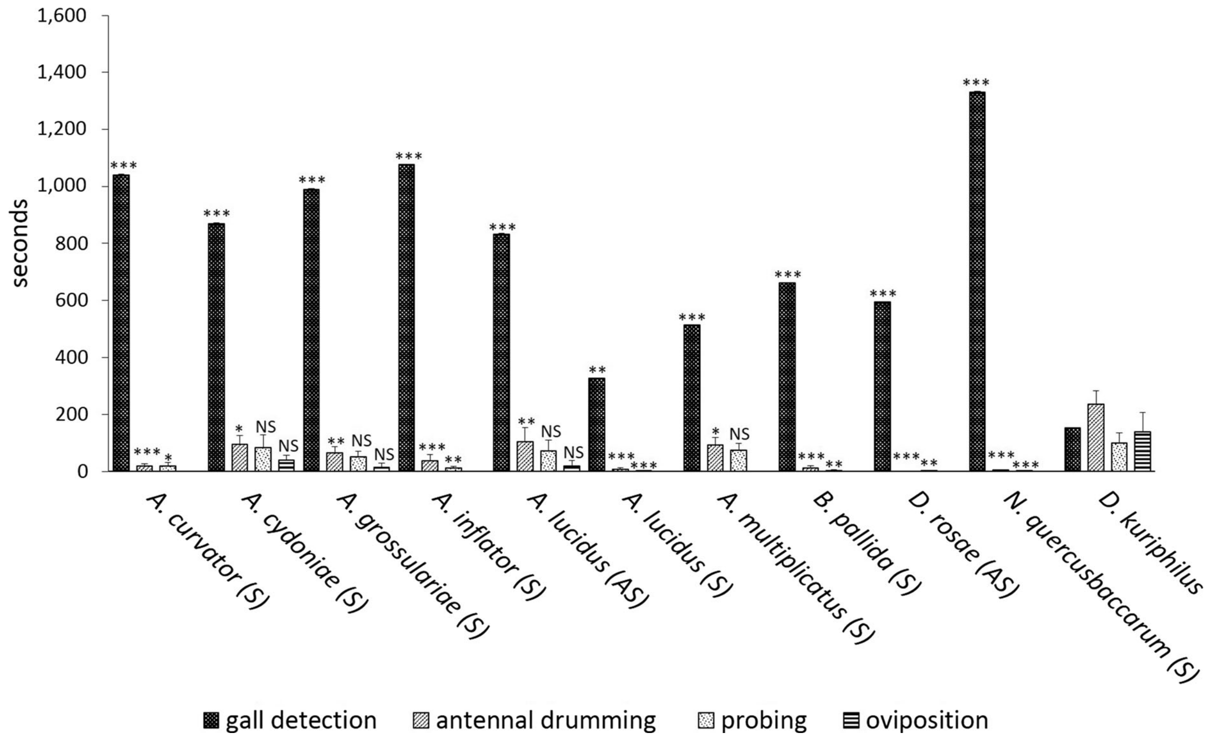

Fig. 2 Average time (+SE) spent for gall detection, antennal drumming, probing and oviposition when a *T. sinensis* female was offered single fresh non-target galls compared to *D. kuriphilus* galls. All the non-target gall species belonged to the sexual generation (S), except for *D. rosae* (asexual generation, AS), and *A. lucidus* (both AS and S). Average of the behaviours recorded for each non-target species was compared with those recorded for *D. kuriphilus*. Averages were compared with non parametric Mann–Whitney *U* test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; *NS* not significant)

the non-target galls was not significantly different from the control (Mann–Whitney *U* test; *A. cydoniae*: Z = −0.840; P = 0.486; *A. grossulariae*: Z = −2.098; P = 0.126; *A. lucidus* asexual generation: Z = −2.042; P = 0.137). The dissection of the galls highlighted the presence of the larvae in the non-target galls on which oviposition occurred, while for all the other species no larva was detected. The cytochrome oxidase I gene obtained from each of the larvae found in the dissected galls 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.

Discussion

A very rich parasitoid community, distributed over six families (Eulophidae, Eupelmidae, Eurytomidae, Ormyridae, Pteromalidae, and Torymidae), was found associated with the galls collected on *Quercus* and *Rosa* by Cynipidae (Cynipini and Diplolepidini). Galls of *B. pallida* and of the asexual generation of *A. lucidus* were shown to be parasitised by more than 20 different chalcid parasitoid species, and *E. brunniventris* and *S. biguttata* were found to be the most generalist species, recorded attacking 32 and 25 different host galls, respectively. These data extend the current knowledge about the parasitoids of cynipid galls in the West Palaearctic available in the literature (Askew et al. 2006, 2013; Rodríguez-Fernández et al. 1997). The Torymidae family represented about 30% of all the specimens collected, confirming the presence, in addition to the native species, of the exotic *T. sinensis*, as well. This parasitoid was first released in 2005 in NW Italy as a BCA of the ACGW. It was known from the literature to be host-specific and able to provide effective biological control in Japan (Moriya et al. 2003).

Due to the risks that BCAs introduced to new countries may pose, causing a decline in species that are not the target pest, in 2004 minimal host testing was performed on *Mikiola fagi* Hartig (Diptera: Cecidomyiidae) and the asexual generations of the oak gall wasps *C. quercusfolii* and *A. kollari* under laboratory conditions (Quacchia et al. 2008). Since no evidence of oviposition on these galls was found, further pre-release studies were skipped in the interest of responding rapidly to the economic threat posed by the ACGW.

Host specificity testing is a key element in predicting the risks of non-target effects of BCAs (Babendreier et al. 2005; Hajek et al. 2016; Hopper 2001), but retrospective studies of previous introductions and their impacts may play an important role (Hopper 2001). Since chestnut and oak trees are commonly present in our forestry environment, ACGW and native cynipids cohabit as well. Since native species most closely related to the targeted species are most likely to be attacked, an extensive study involving chestnut galls and oak galls, with high systematic and ecological affinities, has been carried out to evaluate the specificity of *T. sinensis*.

In this paper the assessment of the non-target impacts of the BCA *T. sinensis* confirmed the observations already carried out in 2013–2014 (Ferracini et al. 2015a), increasing the list of suitable non-target hosts to 15 different oak gall species, including *B. pallida* previously reported. Field evidence for movement of *T. sinensis* to native oak galls was reported in six out of nine of the surveyed regions (Aosta Valley, Emilia Romagna, Latium, Liguria, Piedmont, Tuscany), highlighting how *A. curvator* and *A. inflator* were the most parasitised non-target hosts. In contrast to the field collection, laboratory experiments suggested a lower likelihood of attack. Oviposition was confirmed on *A. cydoniae*, *A. grossulariae* and *A. lucidus* asexual generation, while it was never recorded on *A. curvator*, *A. inflator*, and *B. pallida*, highlighting how physiological/behavioural host-range measured in the laboratory and ecological host-range measured in the field often differ (Hopper, 2001). Host-specificity may in fact be influenced by the test conditions, as already pointed out by Louda et al. (2003) and thus fail to predict the magnitude of non-target risk to native host species in the field. These observations corroborate preliminary host-range laboratory tests during which only brief ovipositor prickings had been observed on *A. cydoniae* and *B. pallida* galls (Quacchia et al. 2014; Ferracini et al., 2015a), and oviposition recorded on *A. curvator* (Ferracini et al. 2015a).

Another concern about the introduction of an exotic natural enemy is the hybridisation with native species, which may represent a further potential environmental impact of biological control. In this case the concern about *T. sinensis* was the likelihood of hybridisation with native *Torymus* spp., as already reported in Japan with the closely related species *T. beneficus* (Yara et al. 2010). Six native *Torymus* species were found in association with galls induced on *Quercus* spp. and rose. Four species were tested in hybridisation trials, namely *T. auratus*, *T. affinis*, *T. flavipes*, and *T. cyaneus*, revealing that no attempted mating or mating behaviour was ever recorded, and confirming previous observations (Quacchia et al. 2014; Ferracini et al., 2015a).

Even if host-range expansion of *T. sinensis* was clearly confirmed in this paper, it is essential to balance this evidence with the impact of the pest, which seriously affected European chestnut growing in the 2000s. The quantification of the risk is a very challenging and difficult activity, and there is not complete agreement on the species that should be considered when evaluating non-target impacts (Hopper, 2001). Although host-range evaluation has to be carried out before the release of a BCA, in this case the pressing need to face the pest, the economic and environmental importance of the Italian chestnut cultivation, and the literature from the area of origin defining *T. sinensis* as monospecific, made the biological control approach the only sustainable and available strategy to manage this invasive pest. Indeed the wasp proved to be a good candidate. It was able to establish, reproduce and spread, having a self-sustaining effect on ACGW and significantly reducing the pest outbreaks in many regions of north-central Italy, and a good control (parasitisation rates up to 98%), and net economic benefits were rapidly achieved (Ferracini et al. 2015a). However, even if the host-range of *T. sinensis* had been evaluated in the field prior to introduction, it would have not reliably predicted its potential because of the plentiful availability of its primary host. Since natural systems are dynamic and BCAs may take a long time to reach equilibrium (e.g. *T. sinensis* took at least 7–8 years to control the ACGW in NW Italy), time is needed to wait until the introduced agent is established before evaluating the impacts of introductions (Hopper 2001), highlighting that the potential adoption of new hosts by a parasitoid is a dynamic process that could take many years (Lopez et al. 2009). The efficacy is the key to understanding and predicting indirect non-target effects of host-specific BCAs, since indirect effects may be proportional to the agent's abundance (Pearson and Callaway 2005).

High population levels developed by BCAs may over-exploit the target species, leading to an unexpected pressure on the non-target hosts. In fact, evidence of emergence by *T. sinensis* from non-target oak galls was recorded only a few years ago, confirming that a host-range may evolve, especially if the BCA is highly successful and finds itself in areas without the target pest or with extremely low level of population. Our finding of 116 *T. sinensis* specimens emerging from oak galls clearly showed this trend, representing a non-target impact, but when evaluating risks *versus* benefits what level of impact should be considered significant? Considering the number of the collected non-target galls and the number of *T. sinensis* specimens emerged, the impact of the exotic BCA appears minimal, occurring at levels that suggest no effect on non-target host densities. Furthermore, many oak gall wasps lay their eggs in clusters, resulting in galls that consist of several larval chambers known as multilocular galls, e.g. *A. cydoniae, A. lucidus*, *B. pallida* (Atkinson et al., 2002; Chinery, 2011; personal observation), which is why the parasitisation rate may probably be overestimated.

Considering the severity index developed by Lynch et al. (2001), a mortality level of at least 40% appears necessary to lead to a serious population-level impact. At the present time considering the number of the galls collected, *T. sinensis* proved to utilise the non-target hosts at a low level, and did not generate sufficient mortality to imply some kind of population-level effect, showing an index not higher than 1 ($< 5\%$ mortality, with no recorded significant population consequences). Only *A. curvator* and *A. inflator* proved to be more parasitised by *T. sinensis*, suggesting a higher suitability for these non-target hosts.

Thus, what would happen if the primary host *T. sinensis* became available again? A spatially explicit model that describes the invasion by the ACGW as well as the effect of the parasitoid *T. sinensis* has recently been developed, showing how the introduction of *T. sinensis* is able to produce a travelling wave that contains the pest. However, the pest is later able to recolonise the empty area left behind the wave, producing an ever-changing pattern of travelling waves. Similarly, in Japan over 25 years, three successive peaks in the population of ACGW were shortly followed by peaks in the population of *T. sinensis* (Paparella et al. 2016). Hence, it is likely that if both populations, the pest and its parasitoid, are able to produce an ever-changing pattern of travelling waves, minor and transitory risks of host-range expansion may occur on non-target hosts. In addition, the recent observations of fecund *T. sinensis* females undergoing a prolonged diapause (Ferracini et al. 2015b; Picciau et al. 2017) may be a response to ACGW shortage and may represent a strategy to stay in synchrony with the primary host, thus reducing the risk of affecting non-target hosts.

Hence, until now, it has been extremely difficult to evaluate the magnitude of adverse effects on non-target galls and whether these effects may be tolerated or be unacceptable, but since a biological approach is aimed at the control of the pest below a bearable threshold, rather than its extinction, it is likely that the presence of the pest, even if at a low level, may imply an occasional feeding on non-target hosts that does not result in changes in their distribution or abundance. Further research is needed to provide a proper assessment of population impact. In particular, investigations are required for the non-target hosts proved to be most frequently parasitised by the introduced BCA (namely *Andricus curvator,* and *A. inflator*), also in the light of the fluctuation trend recently developed by Paparella et al. (2016) both for *T. sinensis* and its primary host *D. kuriphilus*.

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