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

3

4 **Abstract**

5 A post-release study was performed to assess the impact of *Torymus sinensis* (Hymenoptera: Torymidae),
6 a biological control agent of *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae), on native cynipid gall
7 inducers in Italy. In total, 14,512 non-target galls were collected, corresponding to seven genera:
8 *Andricus*, *Aphelonyx*, *Biorhiza*, *Cynips*, *Diplolepis*, *Neuroterus*, and *Synophrus*, and 8,708 chalcid
9 parasitoids were recorded. The Torymidae family accounted for about 30%, and *Bootanomyia* (= *Megastigmus*)
10 *dorsalis*, *Torymus affinis* and *T. flavipes* were the most represented species. A total of 116
11 *T. sinensis* emerged from 15 different oak galls, mainly *Andricus curvator* and *A. inflator*. In controlled
12 conditions, oviposition was recorded on *A. cydoniae*, *A. grossulariae* and *A. lucidus*, while no mating
13 with native congeneric species occurred. This paper confirms the realised host-range expansion by *T.*
14 *sinensis*. Even if it were extremely difficult to evaluate its magnitude, the impact appears minimal, and
15 an occasional feeding with no changes in the distribution or abundance of non-target hosts is expected.

16

17 **Keywords:** *Torymus sinensis*, native chalcid parasitoids, non-target effects, environmental risk
18 assessment, Torymidae, cynipid gall inducers

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22 **Introduction**

23 Alien species are recognised as the second largest threat to biological diversity, the first being habitat
24 destruction. Exotic pests, in the absence of their natural antagonists, may cause unprecedented damage
25 to native biodiversity and the economic impact of invasive pests can be great. The importance of natural
26 enemies for pest control has been known for over a thousand years, reaching all-time highs in the 1960s
27 and 1970s (Hajek et al. 2016). In this context, classical biological control (CBC), the importation and
28 release of an organism outside its natural range to control a pest, attempts to manage invasive pests
29 through the introduction of exotic natural enemies. The release of more than 2,000 species of natural
30 enemies has resulted in the permanent reduction of at least 165 pest species worldwide (Cock et al. 2010;
31 Hajek et al. 2016). The most striking benefit, when compared with any pest control program based on
32 pesticides, is that they can be permanent and self-propagating, and moreover the risks of pesticide
33 resistance are avoided (Boettner et al. 2000; De Clercq et al. 2011; Naranjo et al. 2015; Van Driesche et
34 al. 2010). Many examples of successful CBC can be listed worldwide: the vedalia beetle *Rodolia*
35 *cardinalis* Mulsant (Coleoptera: Coccinellidae) against the cottony cushion scale *Icerya purchasi*
36 Maskell (Hemiptera: Margarodidae) (De Clercq et al. 2011); the egg parasitoid *Anaphes nitens*
37 (Hymenoptera: Mymaridae) against the Australian weevil *Gonipterus scutellatus* (Coleoptera:
38 Curculionidae) (Hanks et al. 2000) in California, (USA); the parasitoid *Epidinocarpis lopezi* De Santis
39 (Hymenoptera: Encyrtidae) for the control of the cassava mealybug, *Phenacoccus manihoti* Matile-
40 Ferrero (Hemiptera: Pseudococcidea) in Africa (Chakupurakal et al. 1994) and the wasp *Neodryinus*
41 *typhlocybae* (Ashmead) (Hymenoptera: Dryinidae) to control the flatid planthopper *Metcalfa pruinosa*
42 (Say) (Homoptera: Flatidae) in North America and Europe (Alma et al. 2005).

43 Nevertheless, the irreversible introduction of a biological control agent (BCA) might bring, with time,
44 negative effects, either direct or indirect, in particular on native non-target species. The outcomes may

45 range on a large scale from negligible to massive effects, the latter especially on vertebrates or molluscs,
46 and are difficult to predict in complex systems (De Clercq et al. 2011; Louda et al. 2003). Concerns about
47 the safety of CBC and its possible consequences have been rising, in particular about their non-transient
48 effects on the environment, such as impacts on natural biodiversity, host switching and dispersal into
49 non-agricultural habitats (De Clercq et al. 2011; Louda et al. 2003; Thomas and Willis 1998).

50 Even if reports of significant environmental impacts are increasing (Boettner et al. 2000; Funasaki et al.
51 1988; Howarth 1991; Louda et al. 2003), and some early CBC programs, especially concerning
52 introduction to islands, have had severe consequences for non-target organisms (Lynch et al. 2001),
53 Hajek et al. (2016) underline how very few cases of quantified negative ecological effects on native
54 species or ecosystem have been documented, or in other cases suspected but not verified, and how no
55 large-scale extinction has yet been reported (Suckling and Sforza 2014). One of the most successful
56 examples of recent European CBC programs is surely represented by the introduction of the Chinese
57 parasitoid *Torymus sinensis* Kamiyo (Hymenoptera: Torymidae), to control the Asian chestnut gall wasp
58 *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) (ACGW). This parasitoid was first
59 released in Italy in 2005, and following the positive Italian experience further release programs were
60 performed in Croatia, France and Hungary, as well as test releases in Spain and Portugal (Ferracini and
61 Alma 2015; Ferracini et al. 2015a; Matošević et al. 2014; Paparella et al. 2016).

62 The urgent need for a full environmental risk assessment and the increasing concern about CBC and its
63 consequences on natural biodiversity, made necessary the evaluation of the possible adoption by *T.*
64 *sinensis* of alternative native hosts. Furthermore, another potential environmental impact of biological
65 control is represented by hybridisation between introduced BCA's and native species. The evidence of
66 hybridisation reported by Yara et al. (2010) between the introduced *T. sinensis* and the native *Torymus*

67 species (i.e. *T. beneficus*) in Japan, highlights the need for knowledge regarding potential adverse
68 consequences towards the native congeneric species.

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

76 On the basis of this recent evidence and with the aim to carry out further research on a broader scale, an
77 exhaustive post-release study was performed. Investigations were carried out in a three-year period
78 (2013-2015) in North-central Italy where *T. sinensis* is established, in order to study the native parasitoid
79 complex associated with non-target hosts, in particular native cynipid species inducing galls on oaks and
80 wild rose. Hence, non-target galls were collected to evaluate the possible adaptation of this exotic wasp
81 as well. Moreover, *T. sinensis* adults were exposed to non-target hosts and native *Torymus* species in no-
82 choice conditions to perform oviposition and hybridisation trials, respectively.

83 **Materials and methods**

84 Gall collection

85 In order to evaluate the potential host-range expansion of *T. sinensis* from chestnut to non-target hosts,
86 collection was carried out in 86 sites in nine Italian regions (Abruzzo, Aosta Valley, Emilia Romagna,
87 Latium, Liguria, Lombardy, Piedmont, Tuscany, and Trentino Alto Adige) in the three-year period 2013-
88 2015. Samplings were performed on common oak (*Quercus robur* L.), downy oak (*Q. pubescens*
89 Willdenow), sessile oak (*Q. petraea* (Mattuschka) Lieblein), Turkey oak (*Q. cerris* L.), and wild rose

90 (*Rosa* spp.). Sampled trees and shrubs were located in mixed forests close to chestnut stands, where a
91 stable *T. sinensis* population had been previously assessed. Additional data about the years of sampling,
92 the regions and the coordinates of the sites are provided in supplementary Table S1. Collection, isolation
93 and maintenance of the galls were performed according to the method described by Ferracini et al.
94 (2015a).

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

100 Insect

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

106 Hybridisation trials

107 Only naïve, six-day-old, unmated individuals were used for the trials for both native parasitoids and *T.*
108 *sinensis*. Courtship and mating behaviour between *T. sinensis* and five native *Torymus* species emerged
109 from oak galls were evaluated; in particular, *T. auratus* Muller (11 males and 19 females), *T. affinis*
110 Fonscolombe (6 males and 9 females), *T. flavipes* Walker (10 males and 4 females), *T. cyaneus* Walker
111 (4 males and 4 females) and *T. geranii* Walker (1 male and 1 female) were used.

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

122 No-choice oviposition trials

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

130 A single fresh non-target gall was offered to a *T. sinensis* female placed on a filter paper sheet inside a
131 Petri dish (diameter 10 cm). For each gall species 15 replications were performed, except for *D. rosae*
132 for which only 10 galls were found. Three behavioural sequences were recorded, as described in Table
133 2. The time spent for gall detection was calculated as the time elapsed from the female's entrance into
134 the arena and the contact with the tested gall. Observations were performed under a stereomicroscope for

135 30 min, using JWatcher[®] software. The average duration of each recorded behavior was compared with
136 the one recorded on *D. kuriphilus* galls. At the end of the trial, the female was left in the Petri dish with
137 the gall for an additional 24 h and then removed. Since eggs might have escaped detection, galls were
138 then stored in a climatic chamber at $24 \pm 2^{\circ}\text{C}$, $50 \pm 10\% \text{RH}$, and a photoperiod of 16:8 (L:D) h for 10
139 days to ease the detection of the parasitoid at larval stage. All the trials were performed under laboratory
140 conditions. To avoid any influence on the behaviour of the parasitoid, chestnut galls were collected in
141 the Trentino Alto Adige region in a site with no presence of *T. sinensis*. On the contrary, since during
142 collection it was not possible to detect previously parasitised galls (e.g. by visual inspection), oak galls
143 were discarded after the trials if any native parasitoid larva was identified by molecular analysis after
144 dissection.

145 Parasitoid identification

146 All the parasitoids emerged from non-target hosts were morphologically identified using specific
147 dichotomous keys (Alkhatib et al. 2014; Askew 1961a; Askew 1961b; Askew and Nieves-Aldrey 2000;
148 de Vere Graham and Gijswijt 1998; Kamijo 1982; Nieves-Aldrey and Askew 1988; Roques and
149 Skrzypczyńska 2003) and by comparison with voucher specimens deposited at the DISAFA-Entomology
150 laboratory, Grugliasco, Italy. Doubtful species and larvae recorded in dissected galls in the no-choice
151 oviposition trials were submitted to DNA extraction and then sequenced for the cytochrome oxidase I
152 (COI) gene following Kaartinen et al. (2010). Parasitoids developed upon inquilines or other insects,
153 such as aculeate Hymenoptera, Coleoptera and Neuroptera, were discarded.

154 Statistical analysis

155 In the hybridisation trials, the time of duration of the following behaviours was recorded: courtship dance,
156 antennal contact, attempted mating and mating. The average time of each behaviour was compared with
157 those recorded in the control trials by non-parametric Wilcoxon signed-rank test ($P < 0.05$). In the no-

158 choice oviposition trials the times that *T. sinensis* females spent in four types of behaviour (gall
159 identification, antennal drumming, probing and oviposition) were recorded, and averages were analysed
160 for each non-target gall and compared with those recorded on ACGW galls (as control) by non-
161 parametric Mann-Whitney U test ($P < 0.05$). All analyses were performed using SPSS version 22.0
162 (SPSS, Chicago, IL, USA).

163 **Results**

164 In total, 14,512 non-target galls were collected, corresponding to seven different genera: *Andricus*,
165 *Aphelonyx*, *Biorhiza*, *Cynips*, *Diplolepis*, *Neuroterus*, and *Synophrus* (Table 3). The galls found most
166 frequently were the sexual generation of *B. pallida* (1,886), and the asexual generations of *Andricus*
167 *quercustozae* Bosc (1,106) and *A. lignicolus* Hartig (1,049).

168 The number of chalcid parasitoids emerged from the non-target galls is reported in supplementary Table
169 S2. In total 8,708 parasitoids from the superfamily Chalcidoidea emerged, and thirty-five species were
170 identified using morphological characters and molecular analyses, distributed over six chalcid families
171 (Eulophidae, Eupelmidae, Eurytomidae, Pteromalidae, Torymidae and Ormyridae). The most frequent
172 species were *Aulogymnus skianeuros* Ratzeburg (964), *Bootanomyia* (= *Megastigmus*) *dorsalis* F.
173 (1,054), and *Sycophila biguttata* Swederus (787). A total of 2,668 native torymid specimens emerged,
174 belonging to three genera: *Bootanomyia* (= *Megastigmus*), *Glyphomerus* and *Torymus*.

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

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

184 Hybridisation trials

185 When native individuals were exposed to *T. sinensis*, only a courtship dance was observed, as shown in
186 Figure 1, while no contact with the antennae, attempted mating or mating behaviour was ever recorded.

187 When male native parasitoids were exposed to a *T. sinensis* female, the average duration of a single
188 dancing event was always significantly lower compared to the control, (Wilcoxon signed-rank test; *T.*
189 *auratus*: $Z = -2.134$, $P = 0.033$; *T. affinis*: $Z = -2.201$, $P = 0.028$; *T. flavipes*: $Z = -42.803$, $P = 0.005$),
190 except for *T. cyaneus* (Wilcoxon signed-rank test; $Z = -1.826$, $P = 0.068$).

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

198 No-choice oviposition trials

199 The average times spent during the entire trial for gall identification, antennal drumming, probing and
200 oviposition are reported in Figure 2. The time needed for gall identification was significantly higher when
201 a *T. sinensis* female was offered single fresh non-target galls, compared to ACGW galls, used as control
202 (Mann-Whitney U test; *A. curvator*: $Z = -3.915$; $P < 0.001$; *A. cydoniae*: $Z = -3.756$; $P < 0.001$; *A.*

203 *grossulariae*: $Z = -3.758$; $P < 0.001$; *A. inflator*: $Z = -4.070$; $P < 0.001$; *A. lucidus* asexual generation: $Z =$
204 -3.332 ; $P < 0.001$; *A. lucidus* sexual generation: $Z = -2.615$; $P = 0.008$; *A. multiplicatus*: $Z = -3.715$; P
205 < 0.001 ; *B. pallida*: $Z = -3.655$; $P < 0.001$; *N. quercusbaccarum*: $Z = -4.143$; $P < 0.001$; *D. rosae*: $Z = -$
206 3.631 ; $P < 0.001$). The time spent for antennal drumming on non-target galls was always significantly
207 lower compared to the control (Mann-Whitney U test; *A. curator*: $Z = -4.015$; $P < 0.001$; *A. cydoniae*:
208 $Z = -2.473$; $P = 0.013$; *A. grossulariae*: $Z = -3.015$; $P = 0.002$; *A. inflator*: $Z = -3.636$; $P < 0.001$; *A.*
209 *lucidus* asexual generation: $Z = -2.586$; $P = 0.010$; *A. lucidus* sexual generation: $Z = -4.478$; $P < 0.001$;
210 *A. multiplicatus*: $Z = -2.249$; $P = 0.023$; *B. pallida*: $Z = -4.232$; $P < 0.001$; *N. quercusbaccarum*: $Z = -$
211 4.550 ; $P < 0.001$; *D. rosae*: $Z = -4.038$; $P < 0.001$). The time spent in the probing activity was significantly
212 lower only for *A. curator*, *A. inflator*, *A. lucidus* sexual generation, *B. pallida*, *N. quercusbaccarum* and
213 *D. rosae* (Mann-Whitney U test; *A. curator*: $Z = -2.185$; $P = 0.037$; *A. inflator*: $Z = -2.769$; $P = 0.010$;
214 *A. lucidus* sexual generation: $Z = -3.629$; $P = 0.001$; *B. pallida*: $Z = -3.324$; $P = 0.002$; *N.*
215 *quercusbaccarum*: $Z = -3.507$; $P = 0.001$; *D. rosae*: $Z = -3.227$; $P = 0.002$), while no differences were
216 measured for the other non-target galls compared to the control (Mann-Whitney U test; *A. cydoniae*: $Z = -$
217 -0.649 ; $P = 0.539$; *A. grossulariae*: $Z = -0.829$; $P = 0.436$; *A. lucidus* asexual generation: $Z = -1.050$; $P =$
218 0.325 ; *A. multiplicatus*: $Z = -0.617$; $P = 0.567$).

219 Oviposition was considered successful when the female spent more than 60 s with the ovipositor inserted
220 in the gall (authors' observation). Oviposition was recorded on three non-target gall species: *A. cydoniae*,
221 *A. grossulariae* and *A. lucidus* asexual generation. The total time spent for the oviposition on the non-
222 target galls was not significantly different from the control (Mann-Whitney U test; *A. cydoniae*: $Z = -$
223 0.840 ; $P = 0.486$; *A. grossulariae*: $Z = -2.098$; $P = 0.126$; *A. lucidus* asexual generation: $Z = -2.042$; $P =$
224 0.137). The dissection of the galls highlighted the presence of the larvae in the non-target galls on which
225 oviposition occurred, while for all the other species no larva was detected. The cytochrome oxidase I

226 gene obtained from each of the larvae found in the dissected galls, was submitted to molecular
227 identification, sequenced and the sequences compared with those in the National Centre for
228 Biotechnology Information (NCBI) sequence database. In all cases, a minimum of 99 % similarity with
229 *T. sinensis*-related sequences was observed.

230 **Discussion**

231 A very rich parasitoid community, distributed over six families (Eulophidae, Eupelmidae, Eurytomidae,
232 Ormyridae, Pteromalidae, and Torymidae), was found associated with the galls collected on *Quercus* and
233 *Rosa* by Cynipidae (Cynipini and Diplolepidini). Galls of *B. pallida* and of the asexual generation of *A.*
234 *lucidus* were shown to be parasitised by more than 20 different chalcid parasitoid species, and *E.*
235 *brunniventris* and *S. biguttata* were found to be the most generalist species, recorded attacking 32 and
236 25 different host galls, respectively. These data extend the current knowledge about the parasitoids of
237 cynipid galls in the West Palaearctic available in the literature (Askew et al. 2006; Askew et al. 2013;
238 Rodríguez-Fernández et al. 1997). The Torymidae family represented about 30% of all the specimens
239 collected, confirming the presence, in addition to the native species, of the exotic *T. sinensis*, as well.
240 This parasitoid was first released in 2005 in NW Italy as a BCA of the ACGW; it was known from the
241 literature to be host-specific and able to provide effective biological control in Japan (Moriya et al. 2003).
242 Due to the risks that BCAs introduced to new countries may pose, causing a decline in species that are
243 not the target pest, in 2004 minimal host testing was performed on *Mikiola fagi* Hartig (Diptera:
244 Cecidomyiidae) and the asexual generations of the oak gall wasps *C. quercusfolii* and *A. kollari* under
245 laboratory conditions (Quacchia et al. 2008). Since no evidence of oviposition on these galls was found,
246 further pre-release studies were skipped in the interest of responding rapidly to the economic threat posed
247 by the ACGW.

248 Host specificity testing is a key element in predicting the risks of non-target effects of BCAs (Babendreier
249 et al. 2005; Hajek et al. 2016; Hopper 2001); but retrospective studies of previous introductions and their

250 impacts may play an important role (Hopper 2001). Since chestnut and oak trees are commonly present
251 in our forestry environment, ACGW and native cynipids cohabit as well. Since native species most
252 closely related to the targeted species are most likely to be attacked, an extensive study involving chestnut
253 galls and oak galls, with high systematic and ecological affinities, has been carried out to evaluate the
254 specificity of *T. sinensis*.

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

270 Another concern about the introduction of an exotic natural enemy is the hybridisation with native
271 species, which may represent a further potential environmental impact of biological control. In this case
272 the concern about *T. sinensis* was the likelihood of hybridisation with native *Torymus* spp., as already

273 reported in Japan with the closely related species *T. beneficus* (Yara et al. 2010). Six native *Torymus*
274 species were found in association with galls induced on *Quercus* spp. and rose; four species were tested
275 in hybridisation trials, namely *T. auratus*, *T. affinis*, *T. flavipes*, and *T. cyaneus*, revealing that no
276 attempted mating or mating behaviour was ever recorded, and confirming previous observations
277 (Quacchia et al. 2014; Ferracini et al., 2015a).

278 Even if host-range expansion of *T. sinensis* was clearly confirmed in this paper, it is essential to balance
279 this evidence with the impact of the pest, which seriously affected European chestnut growing in the
280 2000s. The quantification of the risk is a very challenging and difficult activity, and there is not complete
281 agreement on the species that should be considered when evaluating non-target impacts (Hopper, 2001).
282 Although host range evaluation has to be carried out before the release of a BCA, in this case the pressing
283 need to face the pest, the economic and environmental importance of the Italian chestnut cultivation, and
284 the literature from the area of origin defining *T. sinensis* as monospecific, made the biological control
285 approach the only sustainable and available strategy to manage this invasive pest. Indeed the wasp proved
286 to be a good candidate; it was able to establish, reproduce and spread, having a self-sustaining effect on
287 ACGW and significantly reducing the pest outbreaks in many regions of north-central Italy, and a good
288 control (parasitisation rates up to 98%), and net economic benefits were rapidly achieved (Ferracini et
289 al. 2015a). However, even if the host range of *T. sinensis* had been evaluated in the field prior to
290 introduction, it would have not reliably predicted its potential because of the plentiful availability of its
291 primary host. Since natural systems are dynamic and BCAs may take a long time to reach equilibrium
292 (e.g. *T. sinensis* took at least 7-8 years to control the ACGW in NW Italy), time is needed to wait until
293 the introduced agent is established before evaluating the impacts of introductions (Hopper 2001),
294 highlighting that the potential adoption of new hosts by a parasitoid is a dynamic process that could take
295 many years (Lopez et al. 2009). The efficacy is the key to understanding and predicting indirect non-

296 target effects of host-specific BCAs, since indirect effects may be proportional to the agent's abundance
297 (Pearson and Callaway 2005).

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

310 Considering the severity index developed by Lynch et al. (2001), a mortality level of at least 40% appears
311 necessary to lead to a serious population-level impact. At the present time considering the number of the
312 galls collected, *T. sinensis* proved to utilise the non-target hosts at a low level, and did not generate
313 sufficient mortality to imply some kind of population-level effect, showing an index not higher than 1
314 (<5% mortality, with no recorded significant population consequences). Only *A. curvator* and *A. inflator*
315 proved to be more parasitised by *T. sinensis*, suggesting a higher suitability for these non-target hosts.

316 Thus, what would happen if the primary host *T. sinensis* became available again? A spatially explicit
317 model that describes the invasion by the ACGW as well as the effect of the parasitoid *T. sinensis* has
318 recently been developed, showing how the introduction of *T. sinensis* is able to produce a travelling wave

319 that contains the pest; however, the pest is later able to recolonise the empty area left behind the wave,
320 producing an ever-changing pattern of travelling waves. Similarly, in Japan over 25 years, three
321 successive peaks in the population of ACGW were shortly followed by peaks in the population of *T.*
322 *sinensis* (Paparella et al. 2016). Hence, it is likely that if both populations, the pest and its parasitoid, are
323 able to produce an ever-changing pattern of travelling waves, minor and transitory risks of host-range
324 expansion may occur on non-target hosts. In addition, the recent observations of fecund *T. sinensis*
325 females undergoing a prolonged diapause (Ferracini et al. 2015b; Picciau et al. 2017) may be a response
326 to ACGW shortage and may represent a strategy to stay in synchrony with the primary host, thus reducing
327 the risk of affecting non-target hosts.

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

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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

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 seconds

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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	
<i>Andricus amblycerus</i>	Asexual	Unilocular	7	0	43	50
<i>Andricus aries</i>	Asexual	Unilocular	0	0	124	124
<i>Andricus caliciformis</i>	Asexual	Unilocular	18	0	30	48
<i>Andricus caputmedusae</i>	Asexual	Unilocular	10	166	332	508
<i>Andricus conglomeratus</i>	Asexual	Unilocular	0	0	89	89
<i>Andricus coriarius</i>	Asexual	Multilocular	0	15	103	118
<i>Andricus coronatus</i>	Asexual	Unilocular	0	4	104	108
<i>Andricus curator</i>	Sexual	Unilocular	291	0	713	1,004
<i>Andricus cydoniae</i>	Sexual	Multilocular	173	0	198	371
<i>Andricus dentimitratus</i>	Asexual	Unilocular	0	0	64	64
<i>Andricus foecundatrix</i>	Asexual	Unilocular	82	70	155	307
<i>Andricus galeatus</i>	Asexual	Unilocular	0	2	73	75
<i>Andricus gallaearnaeformis</i>	Asexual	Unilocular	2	0	20	22
<i>Andricus glutinosus</i>	Asexual	Unilocular	8	95	51	154
<i>Andricus grossulariae</i>	Asexual	Multilocular	0	0	16	16
	Sexual	Unilocular	23	0	79	102
<i>Andricus infectorius</i>	Asexual	Unilocular	0	55	0	55
	Asexual	Unilocular	5	0	12	17
<i>Andricus inflator</i>	Sexual	Unilocular	61	1	445	507
<i>Andricus kollari</i>	Asexual	Unilocular	195	36	476	707
<i>Andricus lignicolus</i>	Asexual	Unilocular	226	172	651	1,049
	Asexual	Multilocular	17	51	886	954
<i>Andricus lucidus</i>	Sexual	Multilocular	1	0	7	8
<i>Andricus mitratus</i>	Asexual	Unilocular	0	8	2	10
<i>Andricus multiplicatus</i>	Sexual	Multilocular	39	31	318	388
<i>Andricus polycerus</i>	Asexual	Unilocular	154	21	265	440
<i>Andricus quercustozae</i>	Asexual	Unilocular	34	296	776	1,106
<i>Andricus sieboldi</i>	Asexual	Unilocular	0	0	28	28
<i>Andricus solitarius</i>	Asexual	Unilocular	121	0	44	165
<i>Andricus sternlichti</i>	Asexual	Unilocular	5	80	228	313
<i>Aphelonyx cerricola</i>	Asexual	Unilocular	139	25	129	293
<i>Biorhiza pallida</i>	Sexual	Multilocular	1,470	61	355	1,886
<i>Cynips cornifex</i>	Asexual	Unilocular	137	3	50	190
<i>Cynips disticha</i>	Asexual	Unilocular	0	3	42	45
<i>Cynips divisa</i>	Asexual	Unilocular	229	10	11	250
<i>Cynips quercusfolii</i>	Asexual	Unilocular	191	91	392	674
<i>Diplolepis rosae</i>	Asexual	Multilocular	10	6	61	77
<i>Neuroterus albipes</i>	Asexual	Unilocular	12	0	0	12
<i>Neuroterus anthracinus</i>	Asexual	Unilocular	85	102	375	562
<i>Neuroterus lanuginosus</i>	Asexual	Unilocular	50	3	73	126
<i>Neuroterus minutulus</i>	Asexual	Unilocular	18	0	64	82
<i>Neuroterus quercusbaccarum</i>	Asexual	Unilocular	143	29	409	581
	Sexual	Unilocular	169	8	131	308
<i>Synophrus politus</i>	Sexual	Unilocular	236	10	273	519
Total			4,361	1,454	8,697	14,512

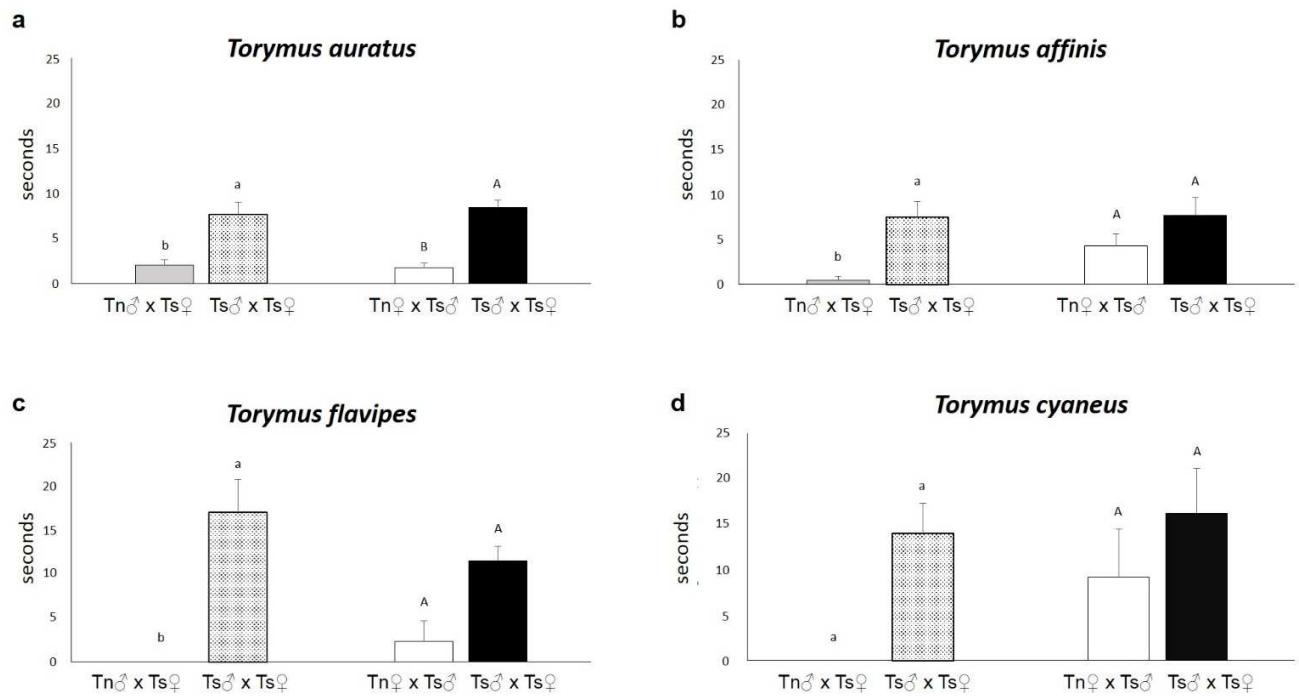


Fig. 1 Average duration (+ SE) of courtship dance events. Interspecific trials were performed exposing four native *Torymus* parasitoids (Tn) [*T. auratus* (a), *T. affinis* (b), *T. flavipes* (c), and *T. cyaneus* (d)] to *T. sinensis* (Ts) specimens of the opposite sex. Interspecific trials using male native parasitoids (Tn♂ x Ts♀) are indicated in grey and compared with their control trials (Ts♂ x 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♀ x Ts♂) are indicated in white and compared with their control trials (Ts♂ x 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, $P < 0.05$)

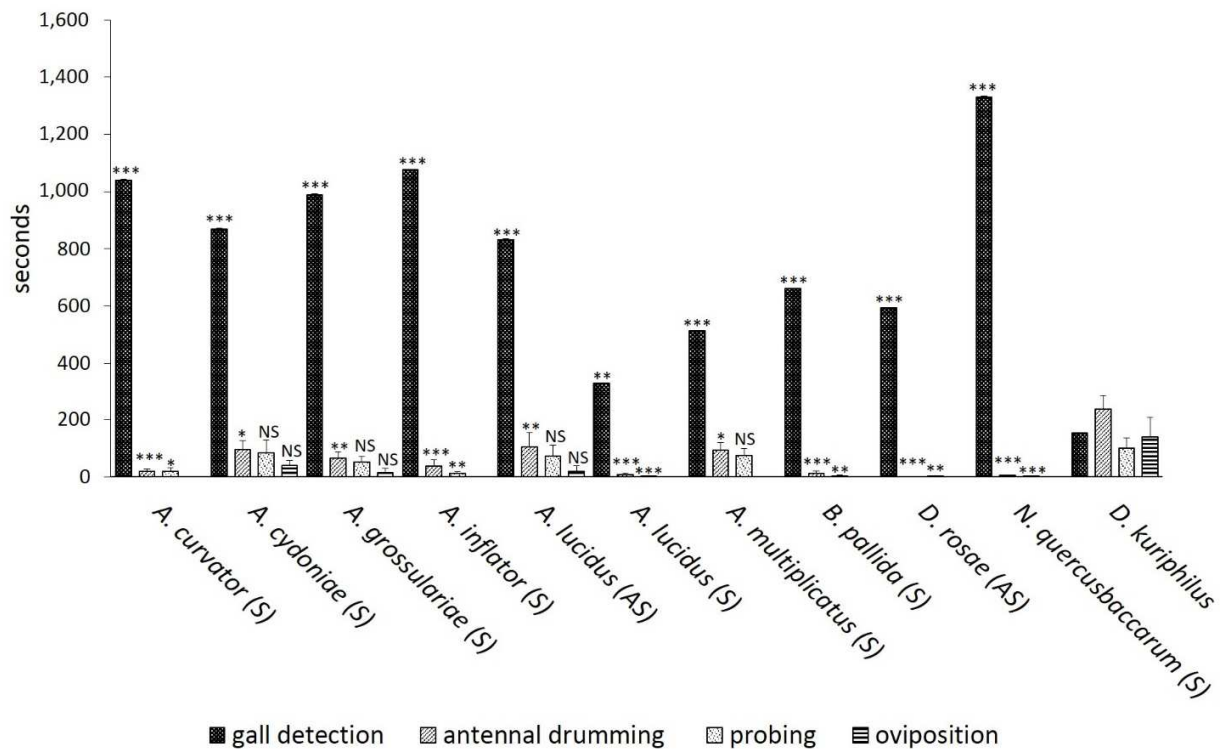


Fig. 2 Average time (+ SE) spent for gall detection, antennal drumming, probing and oviposition when a *T. sinensis* female was offered single fresh non-target galls compared to *D. kuriphilus* galls. All the non-target gall species belonged to the sexual generation (S), except for *D. rosae* (asexual 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 (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; NS=not significant)