UNIVERSITÀ DEGLI STUDI DI TORINO

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

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6   **Abstract**

7   *Torymus sinensis* Kamijo (Hymenoptera: Torymidae) has been released throughout Italy for

8   biological control of the chestnut gall wasp. In response to concern about non-target impacts

9   associated with the introduction of this exotic biological control agent, this study aimed at

10   investigating *T. sinensis*'s host range. In total, 1,371 non-target galls were collected in north-central

11   Italy in a two-year period, representing nine different species. Collections were carried out on

12   common oak, downy oak, sessile oak, Turkey oak, and wild rose.

13   A total of five native torymid species were recorded from the non-target galls (*Megastigmus*

14   *dorsalis*, *Torymus affinis*, *T. auratus*, *T. flavipes*, and *T. geranii*), and three ♂♂ *T. sinensis*

15   individuals emerged from *Biorhiza pallida* galls collected in the field. Under controlled conditions,

16   most of the non-target galls tested were not suitable hosts for oviposition. *T. sinensis* females only

17   laid eggs on *Andricus curvator*. In olfactometer bioassays, higher numbers of *T. sinensis* females

18   showed more interest to the chestnut galls compared to non-target hosts. This data highlights how *T.*

19   *sinensis* has a broader ecological host range than reported in the literature and that it is attracted by

20   non-target hosts other than *D. kuriphilus*.

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

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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

57 hatching the larva feeds ectoparasitically on the host larva, and it pupates in the host larval chamber

58 during winter (Moriya et al., 1990; Ferracini et al. 2015).

59 The introduction of this parasitoid is widely known as one of the typical successful cases of

60 classical biological control in Japan and Italy too (Moriya et al. 2003; Ferracini et al. 2015);

61 however, the risks of the agent concerning potential negative effects on non-target native gall

62 makers mainly present on oaks (*Quercus* spp.) and closely related parasitoids have never been

63 evaluated thoroughly. Murakami et al. (1977) reported *T. sinensis*, among the Chinese parasitoids,

64 as the only species host specific and synchronous with the chestnut gall wasp, but we currently

65 know little about its host range and host specificity in its native or introduced ranges. A few limited

66 host range tests were carried out under laboratory conditions with alternative host galls from

67 *Mikiola fagi* Hartig (Diptera: Cecidomyiidae) , and on asexual generation of the oak gall wasps

68 *Cynips quercusfolii* L. and *Andricus kollari* Hartig (Hymenoptera: Cynipidae) in 2004 (Quacchia et

69 al. 2008), but no oviposition ever occurred. More preliminary tests with seven oak gall species [*A.*

70 *crispator* Tschek, *A. curvator* Hartig, *A. cydoniae* Giraud, *A. grossulariae* Giraud, *A. multiplicatus*

71 Giraud, *Biorhiza pallida* Olivier, and *D. cerriphilus* Giraud (Hymenoptera: Cynipidae)] were also

72 performed in 2014, supporting the specificity thesis further (Quacchia et al. 2014).

73 In response to concern about non-target impacts associated with the introduction of this exotic

74 biological control agent, the EFSA Panel on Plant Health established a new alternative host species

75 list for testing the host-specificity of *T. sinensis*, comprising galls which may be more susceptible to

76 attack during the period that females are searching for hosts, such as *A. curvator* sexual generation;

77 *A. cydoniae* sexual generation; *A. grossulariae* sexual generation; *A. inflator* sexual generation; *A.*

78 *lucidus* sexual generation; *A. multiplicatus* sexual generation; *B. pallida* sexual generation; *D.*

79 *cerriphilus* sexual and asexual generation; *Neuroterus quercusbaccarum* (L.) sexual generation)

80 (EFSA Panel On Plant Health 2010).

81 The present study aimed at investigating *T. sinensis*'s host range, with particular regard to the oak

82 galls species listed by EFSA Panel On Plant Health (2010). No choice trials were carried out

83 applying recently-developed protocols for host/prey range testing, considering the hypothesis that

84 species most closely related taxonomically and ecologically to the target are more likely to be

85 utilized as hosts by the biological control agent being tested (Kuhlmann et al. 2006; van Lenteren et

86 al. 2006). Furthermore, olfactometer bioassays were performed to assess the attractiveness of

87 volatiles for *T. sinensis* females; experiments were conducted to test the response of the parasitoid

88 comparing chestnut galls, reported in literature as the only target host, *versus* non target oak galls.

89 **Materials and methods**

90 Insect

91 All *T. sinensis* used in the trials emerged from parasitized chestnut galls randomly collected, by

92 hand from low branches (from ground level to 2 m high) and with the aid of lopping shears from the

93 medium-high tree crown (from 2 to 5 m high), once a year, in winter, both in 2013 and 2014 in

94 chestnut orchards in Piemonte region, where the parasitoid was first released in 2005 and then

95 successfully established. The galls were kept in cardboard boxes outdoors from January to April

96 until emergence of the parasitoids (Tmin=-1.9, Tmax=16.3°C, RHmin=67.8=, RHmax=78.8% in

97 2013; Tmin=0.8°C, Tmax=19.3°C, RHmin=64.5=, RHmax=81.1% in 2014) . Newly emerged

98 females were fed every 48 h with drops of honey on cardboard and individually kept in glass tubes

99 (120 mm in height by 18 mm in diameter), with no previous contact with a host, in a climatic

100 chamber at 15 ±1 °C, 60±5% RH, and a photoperiod of 16:8 (L:D) h.

101 Gall collection

102 Non-target galls were collected in 4 regions (2 sites per region): Liguria [Borzonasca (GE)

103 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)

104 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)

105 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

106 [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).

107 Collections were carried out on common oak (*Quercus robur* L.), downy oak (*Q. pubescens*

108 Willdenow), sessile oak (*Q. petraea* (Mattuschka) Lieblein), Turkey oak (*Q. cerris* L.), and wild

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109 rose (*Rosa* spp. L.). The selection of non-target oak hosts was based on the species list for host-

110 specificity testing established by EFSA Panel On Plant Health (2010), and host galls from the genus

111 *Rosa*, since their abundance in the wood, were tested as well as suggested by Gibbs et al. (2011).

112 Investigations were carried out over a 2-year period (2013–2014) in all regions and in the same

113 sites. Withered galls were collected in January-February in order to verify if *T. sinensis* may emerge

114 from non-target hosts, and fresh galls were collected in April-May (during *T. sinensis* emergence)

115 both on non-target hosts and chestnut trees (used as a control), to perform oviposition trials and

116 olfactometer bioassays under controlled conditions.

117 Sampled trees and shrubs were located in mixed forests close to infested chestnut stands where *T.*

118 *sinensis* was previously released in order to evaluate a potential shift from chestnut to non-target

119 hosts. In the Table 1 the years of release and the coordinates of *T. sinensis* release points are

120 provided. Collections were made by hand from low branches (from ground level to 2 m high) and

121 with the aid of lopping shears from the medium-high tree crown (from 2 to 5 m high). Galls were

122 stored in plastic bags, transferred immediately to the laboratory, and identified using voucher

123 specimens deposited at the DISAFA-Entomology laboratory.

124 Withered galls were individually isolated in plastic containers (70 mm in height by 55 mm in

125 diameter) with a fine-gauze lid, and stored outdoors within 24 hrs from collection. Containers were

126 checked weekly, and then daily after the first parasitoid emerged. Fresh galls were kept in climatic

127 chamber at 15 ±1 °C, 60±5% RH, and a photoperiod of 16:8 (L:D) h and used in the trials within 24

128 hrs from collection. Containers were kept outdoors from January to December until emergence of

129 the parasitoids (Tmin=-1.9, Tmax=29.1°C, RHmin=61.7%=, RHmax=83.4% in 2013; Tmin=0.8°C,

130 Tmax=26.3°C, RHmin=63.0%=, RHmax=89.7% in 2014) .

131 <u>No choice oviposition trials</u>

132 These tests, based on the 'no choice black box test' (van Lenteren et al. 2006), aimed to test *T.*

133 *sinensis*'s host range, investigating the parasitoid's ability to develop in non-target hosts. No choice

134 exposures to non-target hosts were conducted in an enclosed arena in order to maximise the

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135   likelihood of non-target attack. Four fresh oak gall species included in the EFSA list were tested in

136   2013 (*A. curvator*, *A. grossulariae*, *B. pallida*, and *N. quercusbaccarum*), and only two in 2014 (*A.*

137   *curvator*, and *B. pallida*).

138   A single fresh non-target gall was offered to a 6-d-old naïve *T. sinensis* female placed on a wet filter

139   paper inside a Petri dish (diameter, 10 cm) in order to obtain data on its behaviour in relation to

140   parasitism, with a minimum of 20 replications for each gall species. Observations were performed

141   using a stereomicroscope for 1 hour. Three behavioural sequences were recorded: host location,

142   defined as a walk on the gall locating the host through vibrotaxis, attempted oviposition, defined as

143   the attempted insertion of the ovipositor, and oviposition, defined as successful insertion of the

144   ovipositor followed by the pumping action of the abdomen.

145   At the end of the observation the female was then removed from the arena and individually isolated

146   in a Petri dish containing a fresh unparasitised chestnut gall as control, and the three behavioural

147   sequences were recorded following the same procedure described above. All the tested galls were

148   individually stored in glass tubes (120 mm in height by 18 mm in diameter), and then dissected with

149   the aid of a scalpel using a stereomicroscope. Since eggs may have escaped detection, galls were

150   stored in a climatic chamber at 24±2°C, 50±10% RH, and a photoperiod of 16:8 (L:D) h for 10 days

151   to ease the detection of the parasitoid at larval stage. All the trials were performed in laboratory

152   conditions. To avoid any influence in the behaviour of the parasitoid, chestnut galls were collected

153   in Alto Adige region in a site with no presence of *T. sinensis*. On the contrary, since during

154   collection it was not possible to detect previously parasitised galls (e.g. by visual inspection), oak

155   galls were discarded after the trials if any native parasitoid larva was identified by molecular

156   analysis after dissection.

157   <u>Olfactometer bioassays</u>

158   In the olfactometer bioassays, 6-d-old *T. sinensis* females were used to assess their olfactory

159   responses to the odours of the chestnut gall (used as a control) and 6 non-target galls (*A. curvator*,

160   *A. cydoniae*, *A. grossulariae*, *A. multiplicatus*, *B. pallida*, *N. quercusbaccarum*) as alternative hosts.

161   Before the trials, the insects were individually kept at room temperature without any host in a glass

162   tube for 18 h with a humid cotton cap and microdrops of honey to acclimate the wasps to the

163   experimental conditions. The bioassays were carried out in a horizontal Y-shaped Pyrex tube

164   following the procedure described for another wasp, *Necremnus* spp. (Ferracini et al. 2012). The air

165   flow was provided by an air pump (Air 275R, Sera, Heinsberg, Germany) and then filtered in an

166   activated CO_2 filter, regulated with a flowmeter at 2.5 liters/min (EK-2NRK, Comer, Bologna,

167   Italy) and humidified in a 1-liter water bubbler half filled with deionized water. After the air flow

168   was established, a single parasitoid female was introduced into the entry arm. Each female was

169   observed until she had moved at least 2 cm up one of the side arms or until 10 min had elapsed. For

170   each test the same odour sources were used while concerning the wasps, a female was evaluated

171   only once to prevent any behaviour conditioned by experience. The odour sources chosen by

172   females that responded were recorded. Thirty responses were recorded for each pair of odour

173   sources. After testing five females, the odour sources were switched between the left-hand and

174   right-hand side arms to minimize any spatial effect on choices. The Y-tube and cameras were

175   cleaned with mild soap and alcohol (70% v) and sterilized in an autoclave at 120°C for 20 min. The

176   olfactory bioassays were conducted at 24±2°C, 50±10% RH, and 250±10 lux.

177   Parasitoid identification

178   Among all the parasitoids emerged from non-target hosts, only the torymid species were

179   morphologically identified using specific dichotomous keys (Kamijo 1982; de Vere Graham and

180   Gijswijt 1998) and by comparison with voucher specimens deposited at the DISAFA-Entomology

181   laboratory. Doubtful species and larvae recorded in dissected galls in the no choice oviposition

182   trials were submitted to DNA extraction and then sequenced for the cytochrome oxidase I (COI)

183   gene following Kaartinen et al. (2010).

184   Statistical analyses

185   In the behavioural trials the numbers of times that *T. sinensis* females engaged in three types of

186   behaviour (host location, attempted oviposition, and oviposition) were recorded, and means were

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187 analyzed for each non-target gall to *D. kuriphilus* gall (as control) by paired t-tests for dependent

188 samples. In the olfactory bioassays, the responses of parasitoid females were analysed by a chi-

189 square test. The null hypothesis was that parasitoid females had a 50:50 distribution across the two

190 odour sources. All analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL).

191 **Results**

192 In total, 1,371 non-target galls were collected over the 2-year period, corresponding to 4 different

193 genera: *Andricus*, *Biorhiza*, *Diplolepis,* and *Neuroterus* (Table 2). The galls found most frequently

194 were the sexual generations of *B. pallida* (856), while only 3 galls from *A. grossulariae* and 1 gall

195 from *A. lucidus* were recorded. A total of 707 native torymid specimens emerged from the isolated

196 galls, belonging to 5 species: *Megastigmus dorsalis*, *Torymus affinis*, *T. auratus*, *T. flavipes*, and *T.*

197 *geranii* (Table 3). The most frequent species was *T. flavipes* (381 specimens), while *M. dorsalis* (6

198 specimens), and *T. geranii* (3 specimens) were recorded sporadically. *B. pallida* galls proved to be

199 parasitized by all the parasitoid species recorded, except for *M. dorsalis*, which was recorded

200 emerging from galls of *A. cydoniae*, *A. lucidus*, and *A. multiplicatus*. *T. geranii* emerged only from

201 *B. pallida* galls, and the only species recorded emerging from *D. rosae* was *T. auratus*.

202 In addition to native torymid species, in 2013 a total of 3 ♂♂ *T. sinensis* individuals emerged from

203 non-target *B. pallida* galls collected in the Piemonte region in both surveyed sites (2 ♂♂ from

204 Pianfei and 1 ♂ from Molare). The cytochrome oxidase I gene obtained from the specimens

205 submitted to molecular identification was sequenced and sequences were compared with those in

206 the National Center for Biotechnology Information (NCBI) sequence database. In all cases, a

207 minimum of 99 % similarity with *T. sinensis*-related sequences was observed.

208 No other emergence of the exotic parasitoid was recorded for the other non-target oak galls nor for

209 *D. rosae* during the surveyed period (Table 3). All the torymid species emerged from the withered

210 non-target galls were collected between April and May, depending on the site.

211 <u>No choice oviposition trials</u>

212 In the no choice oviposition trials all the behavioural traits recorded on non-target and target hosts

213 are reported in Table 4. In the close confinement imposed by the experimental design, *T. sinensis*

214 females responded to all non-target and target species by locating and investigating the hosts. The

215 number of host location events was significantly lower for *A. curvator* both in 2013 and 2014 (t

216 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*

217 (t test=-3.14; df=19; P=0.005), and *N. quercusbaccarum* (t test=-4.36; df=19; P<0.001) compared to

218 the control, while for *B. pallida* no significant differences were revealed (t-test=-0.82; df=19;

219 P=0.42 in 2013; t-test=-1.18; df=19; P=0.25 in 2014). Attempts of oviposition were observed when

220 the parasitoid was offered *B. pallida* galls both in 2013 and in 2014 (t-test=1.83; df=19; P=0.08 in

221 2013; t-test=1.79; df=19; P=0.09 in 2014), but no oviposition ever occurred.

222 Only in 2014 both host location and oviposition was observed when the parasitoid was offered a

223 non-target gall. In fact, 6 *T. sinensis* females out of the 20 tested showed interest in a non-target

224 host, laying eggs in *A. curvator* galls (each female laid one egg per gall), although this was

225 significantly lower that the number of *T. sinensis* females that subsequently oviposited on the

226 control, *D. kuriphilus* galls (t-test=-3.25; df=19; P=0.004).

227 In the non-target galls tested no native parasitoid was detected by molecular analysis carried out

228 after dissection. In the control trials oviposition occurred in 96% of the chestnut galls tested.

229 The cytochrome oxidase I gene obtained from each of the larvae found in the dissected galls, both

230 on non-target and target hosts, was submitted to molecular identification, sequenced and the

231 sequences compared with those in the National Centre for Biotechnology Information (NCBI)

232 sequence database. In all cases, a minimum of 99% similarity with *T. sinenisis*-related sequences

233 was observed.

234 Olfactometer bioassays

235 In the olfactometer bioassays all the *T. sinensis* females tested responded by making a choice within

236 the fixed time. Higher numbers of *T. sinensis* females were attracted to the chestnut galls compared

237 to non-target hosts. In particular, significant differences in the responses of adults were found when

238   chestnut gall was compared to *A. cydoniae* (χ^2=6.53; df=1; P=0.01), and *A. grossulariae* (χ^2=13.33;

239   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;

240   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

241   significant differences were observed (Figure 2).

242   **Discussion**

243   In recent years there has been growing concern about the potential or actual threat presented by

244   alien entomophagous biological control agents to populations of native non-target arthropod species

245   (López-Vaamonde and Moore 1998). The use of tests to assess plants as potential hosts for

246   herbivorous insects began over 70 years ago and has long been routine. In contrast, interest in

247   estimating parasitoid and predator host ranges has lagged considerably behind (van Driesche and

248   Murray 2004).

249   A full environmental risk assessment relies on the identification and evaluation of potential risks

250   associated with natural enemy release and the development of a plan to minimize them. That is why

251   in a classical biological program it is extremely important, prior to releasing the exotic natural

252   enemy, to identify, assess and weigh all adverse and beneficial effects in a risk-cost benefit

253   assessment (Gibbs et al. 2011).

254   The set of species that can support the development of a parasitoid or serve as prey for a predator—

255   observed under laboratory conditions exclusively—is defined as the fundamental host range of a

256   potential agent, also termed the physiological host range. In contrast, the ecological host range is

257   defined as the current and evolving set of host species actually used for successful reproduction in

258   the field (Onstad and Mcmanus 1996; Haye et al. 2005). However, assessment of the host range of a

259   biological control agent in the laboratory often yields a significantly broader fundamental host

260   range in comparison to the ecological host range (Haye et al. 2005), overestimating the field host

261   range. Generally, the results of the host specificity study constitute a key factor in the risk analysis

262   performed before an exotic beneficial arthropod can be safely utilized as a biological control agent.

263   In the last decade annual chestnut production in Italy underwent several drops, mainly ascribed to

264   adverse climatic conditions and pests. In particular, the Asian chestnut gall wasp has been

265   responsible for a severe reduction in fruiting, with yield losses estimated to reach between 65% and

266   85% in northern Italy (Bosio et al. 2013; Battisti et al. 2014), with a heavy economic impact on

267   Italian chestnut production.

268   From the first report of this pest in Italy in 2002, following the successful experiences in Japan and

269   North America, and due to the severity of the problem, pros and cons in the release of the exotic

270   parasitoid were balanced, and biological control was considered the only economically and

271   environmentally sustainable solution to deal with the pest promptly, since in the literature

272   alternative approaches (e.g. chemical treatments, resistant varieties) were all found infeasible. In its

273   native distribution, *D. kuriphilus* populations are controlled by natural enemies. In all the countries

274   invaded by the pest a rich parasitoid community has been reported, but the attack rates have

275   remained low (typically less than 2%) (Aebi et al., 2007; Gibbs et al., 2011; Quacchia et al., 2013).

276   Introductions of exotic organisms carry with it some unknown level of environmental risk, but these

277   risks must be weighed against the consequences of not initiating biological control, which can also

278   include serious environmental as well as economic consequences (Heimpel et al., 2004). The

279   releases of *T. sinensis* carried out aided in restoring a habitat to similar conditions as those observed

280   prior to the pest introduction, representing a large benefit for the chestnuts and chestnut growers.

281   Even though the host range of *T. sinensis* has never been studied or tested in detail in either its

282   native or introduced ranges over a long period of time, the parasitoid was considered specific to *D.*

283   *kuriphilus* (Murakami et al. 1977; Quacchia et al. 2014).

284   Nevertheless the host range of an apparently strictly monophagous parasitoid species may not be

285   constant, either in space or time; it could expand in environments with greater diversity and hence

286   have a larger number of new potential hosts (López-Vaamonde and Moore 1998). Attacking non-

287   target hosts is of concern due to the potential harm that exotic natural enemies may impose on

288   native or beneficial exotic species (Nadel et al. 2009). However, the risk to non-target species is

289 complex and difficult to estimate; guidelines for appropriate host range tests have been proposed by

290 the EFSA Panel on Plant Health (2010). Following this suggestion, our approach was to assess *T.*

291 *sinensis*'s capacity to attack and reproduce on non-target gall species inhabiting common habitat

292 with *Castanea* trees.

293 Even if, to date, no severe non-target effects have ever been reported in the literature after the

294 release of *T. sinensis*, this paper represents the first report of potential negative effects on non-target

295 native galls makers mainly present on oaks (*Quercus* spp.) by introducing the exotic parasitoid *T.*

296 *sinensis* as a biological control agent for the chestnut gall wasp, *D. kuriphilus*. In contrast to

297 Quacchia et al. (2008; 2014), who confirmed a high level of specificity for *T. sinensis* on the basis

298 of a set of non-target species tested, our study highlights how *B. pallida* oak galls proved to be

299 successfully parasitized. Even if the case record was low (3 galls parasitised by *T. sinensis* out of

300 856 collected in the field), this finding suggests that this oak gall species is a suitable host for the

301 exotic parasitoid. In 2013 the emergence of *T. sinensis* was recorded only from *B. pallida* galls

302 collected in both surveyed sites of Piemonte region; that is why, due to the considerable presence of

303 *B. pallida* in our environment, a mass collection of this gall species was performed in 2014, but no

304 other emergence was recorded. In the laboratory the conditions under which non-target tests are

305 conducted may also limit interpretation of the host range. Test arenas confine the parasitoid with the

306 host and may force encounters with non-target hosts, increasing the probability of the parasitoid

307 accepting completely factitious hosts (Mason et al. 2011). Nevertheless, in the no choice oviposition

308 trials, when they were offered to *T. sinensis* females oviposition only occurred in 2014 on *A.*

309 *curvator*; since galls were dissected to detect the presence of the larvae, no data is available about

310 their potential development to the adult stage. Some probing attempts were recorded on *B. pallida*,

311 but no oviposition ever occurred.

312 And in general, *T. sinensis* proved to be more attracted by chestnut galls compared to non-target

313 hosts, showing a similar responsiveness in the olfactometer bioassays as well. Statistical differences

314 were observed only for *A. cydoniae* and *A. grossulariae*, but the interest showed by the parasitoid

315 for *A. curvator*, *A. multiplicatus*, *B. pallida*, and *N. quercusbaccarum* have to be further

316 investigated.

317 In the oviposition trials, all the parasitoid females were naïve and tests were conducted only under

318 no choice conditions. In accordance with Withers and Brown (2005), no choice tests with both

319 naïve and oviposition-experienced females should be performed because it has been shown that

320 oviposition experience may either reduce or enhance responsiveness. Furthermore, since parasitoids

321 can display wider host ranges in choice tests, it would be useful to set up choice trials where the

322 parasitoid is given a choice of host and non-target host for a more accurate prediction of potential

323 host range. In fact, parasitoid response is generally expected to be biased toward the familiar host,

324 especially after the parasitoid successfully oviposits in it (Nadel et al. 2009).

325 In this study we confirmed that *T. sinensis* has a broader physiological host range than reported in

326 the literature and determined that it may be attracted by non-target hosts other than *D. kuriphilus*.

327 The assessment of risk requires consideration of the likelihood and magnitude of an effect and

328 evaluation of risk management priorities (Moeed et al., 2006). Methods for quantifying the

329 magnitude and spatiotemporal scale of impact of exotic natural enemies on populations of native

330 insects are crucial to advance current risk assessment (Wyckhuys et al., 2009), however, the

331 incidence of these host shift in the complex chestnut-oak is currently difficult to be quantified in the

332 natural environments.

333 Over the 2-year period, in order to monitor the potential emergence of *T. sinensis*, 8 oak gall species

334 suggested in the EFSA list were collected, while in the oviposition trials 4 non-target species out of

335 9 were tested. At present, research is ongoing to test *A. cydoniae*, *A. inflator*, *A. lucidus*, *A.*

336 *multiplicatus*, and *D. cerriphilus* in controlled conditions as possible hosts for *T. sinensis*, since they

337 provide the closest phenological match to the flight period of the parasitoid (i.e. between April and

338 May in Italy) (EFSA Panel On Plant Health Aebi et al., 2011). Further investigations need also to

339 be performed on *B. pallida*, given the emergence of the parasitoid from galls collected in the field

340 and the interest showed by some females in the oviposition trials.

341 Food availability is an important aspect that may influence the biological traits of many arthropods.

342 Recently a novel insight concerning *T. sinensis'*s life cycle was highlighted in this regard: a

343 prolongation of diapause was in fact reported. Even if it is hard to speculate what are the factors that

344 triggered this response, this may be read as an adaptive value to protect the population against the

345 yearly fluctuation in food supply (Ferracini et al. 2015). At present, there is growing evidence that

346 the *T. sinensis* parasitism rate is dramatically increasing in some Italian regions, almost reaching

347 98% in old release sites (Bosio et al., 2013), and parasitisation on non-target hosts was recorded

348 only in sites where a stable population of the exotic parasitoid is present, (Piemonte region), since

349 the first releases date back to 2005. Hence, a host-shift to oak galls may be due to the need to find

350 another suitable host since populations of the Asian chestnut gall wasp have recently declined

351 significantly (Ferracini et al. 2015), and even though the frequency of cases of observed non-target

352 impacts were small, major effects on non-target galls populations could be expected to be

353 detectable. Hence, longer term studies are necessary to allow more precise conclusions to be drawn

354 on non target impacts, that is why an exhaustive research about all potential non-target galls and

355 their phenology is needed in order to better understand the relationship between the exotic

356 parasitoid and native biocoenoses. At the same time, since in this paper four native *Torymus* species

357 emerged from non target galls during *T. sinensis* flight period as well, and in literature five native

358 species [*T. auratus*, *T. erucarum* (Schrank), *T. flavipes*, *T. geranii*, and *T. scutellaris* (Walker)] are

359 reported from chestnut galls (Alma et al., 2015), an evaluation of the potential for hybridization

360 between these congeneric species is also required in order to have a comprehensive knowledge of

361 the environmental risk to non-target species that *T. sinensis* may pose to native biodiversity.

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472   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

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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

505 Table 3 Numbers of native torymid species and the exotic *Torymus sinensis* (in bold) emerged from

506 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

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515   Table 4 Mean number (±SE) of host location, attempted oviposition, and oviposition events

516   engaged in by *T. sinensis* females comparing non-target oak galls[+] and *D. kuriphilus* galls (control)

517   recorded during 1 h observation periods in no choice oviposition trials over a two-year period

518   (2013–2014). Means were compared for each non-target species using a paired t-tests for dependent

519   samples; P<0.05 *; P<0.01 **; P<0.001***.

Year	Non-target species compared to the control	No.	Host location	Attempted oviposition	Oviposition
	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***
2013	*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
	Andricus curvator	20	0.35± 0.11**	0.00	0.30 ± 0.11**
2014	*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

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

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522   Figure 1 Location of the sampling sites (black dots). The inset indicates the location of the four

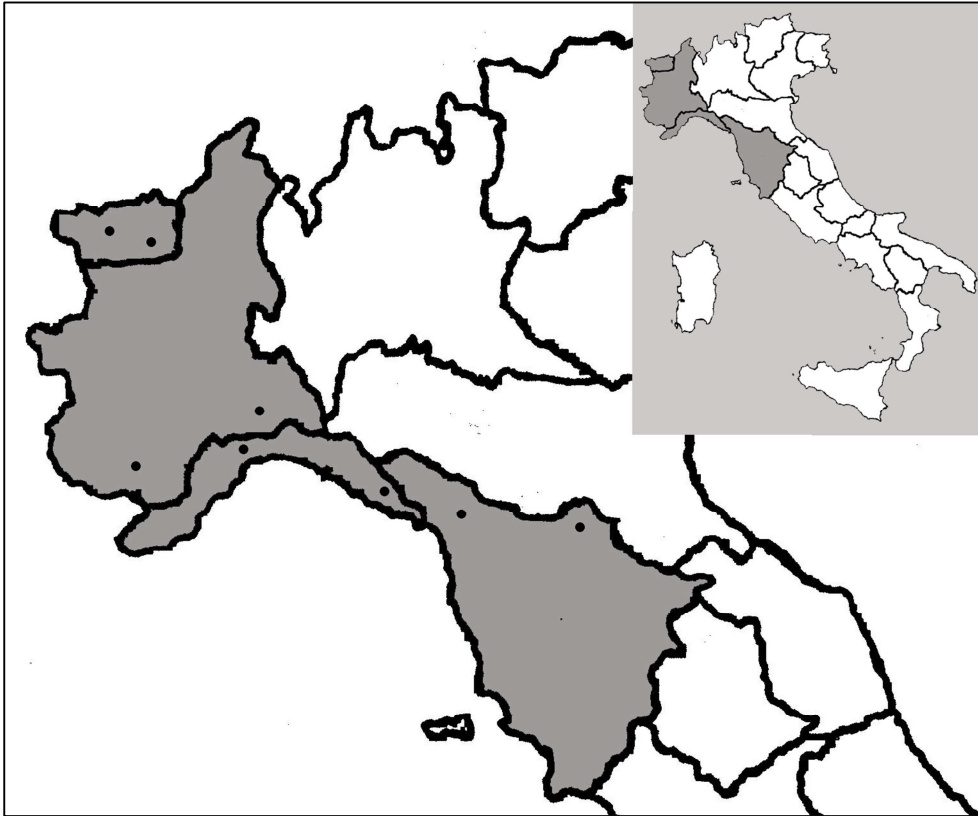523   surveyed regions in Italy.

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525   Figure 2 Responses of *T. sinensis* (number of responding females in bars) in a Y-tube olfactometer

526   to the odours of chestnut gall and non-target galls. Numbers in bars represent individuals that

527   moved toward the volatiles. Chi-square statistics (* P <0.05; df=1) tested the hypothesis that the

528   distribution of side arm choices deviated from a null model where odour sources were chosen with

529   equal frequency.

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