



## *Torymus sinensis* against the invasive chestnut gall wasp: Evaluating the physiological host range and hybridization risks of a classical biological control agent

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### HIGHLIGHTS

- *T. sinensis* can attack native species under laboratory and field conditions.
- Molecular and mating data show that there is no hybridization with the native species.
- We detected the presence of the alien species *Torymus beneficus* in Spain.
- Decisions on biological control of *D. kuriphilus* by *T. sinensis* are discussed.

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### ABSTRACT

**Background:** A common strategy to limit the negative impact of biological invasions is biological control through the release of specialized alien natural enemies. However, biological control plans are not without risks, which include parasitism of native hosts and hybridization with related native species, particularly those that are potential natural enemies of the invasive species. Here, we evaluate these potential risks resulting from the introduction of the parasitoid wasp *Torymus sinensis* (Hymenoptera: Torymidae) in Europe to control the invasive Asian chestnut gall wasp *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae).

**Results:** We found that, under laboratory conditions, the physiological host range of *T. sinensis* includes several native non-target species of Cynipidae, with oviposition observed on the galls of 8 of the 11 species tested. However, physiological host range of *T. sinensis* appears to be limited under field conditions, as we observed only one parasitized gall of *Andricus curvator* in the field. Regarding hybridization, inter-species mating between *T. sinensis* and its phylogenetically closest native *Torymus* species was not observed in the laboratory. Moreover, discordance between nuclear (*ITS2*) and mitochondrial (*cox1*) data does not support the presence of genetic introgression, suggesting that hybridization between *T. sinensis* and native *Torymus* species does not occur. In addition, we cite and discuss the unexpected presence of one individual of the related alien species *Torymus beneficus* in Spain.

**Conclusion:** Our results suggest that *T. sinensis* may negatively impact several non-target species, highlighting the need for careful monitoring of the extent of such undesired behaviour and its effects on the native fauna.

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## 1. Introduction

Biological invasions are considered one of the main human-induced drivers of ecological disturbance and subsequent biodiversity loss (Lodge, 1993; Schmitz & Simberloff, 1997; IPBES, 2019). Invasions directly impact the conservation of biological communities by producing numerous, sometimes irreversible, changes in invaded areas, which in turn, affect ecosystems and native biological communities (Vitousek et al., 1996; Messing & Wright, 2006; Roy & Wajnberg, 2008). Biological invaders include pests that can also cause substantial economic losses through decreases in the production of crops, fruits, or wood, among other ecosystem services (Pimentel, 2014; Hall, 2019). Numerous techniques have been developed to limit the economic damage of such pests, including biological control strategies (DeBach & Rosen, 1991). Classical biological control agents have a co-evolutionary relationship with their target pest and numerous studies have demonstrated the success of this type of biological control practice (DeBach & Rosen, 1991; Landis et al., 2000) and numerous pest management practices have been developed using natural enemies (e.g., Beddington et al., 1978; Hokkanen & Sailer, 1985; van Lenteren, 2000).

However, classical biological control implies the introduction of foreign species and, in some cases, host specificity testing has not been adequately carried out to raise awareness of such risks and mitigate potential impacts. Exotic biological control agents can decrease the abundance and limit the distribution of non-target native species through 1) direct impacts due to predation or parasitoidism; 2) indirect impacts such as competition or displacement; 3) novel disease introduction; 4) biodiversity loss due to hybridization; and 5) impacts on other introduced biological control agents (van Driesche & Hoddle, 2016; van Lenteren et al., 2006). Indeed, there are many examples of biological control agents causing unexpected damage to native biological communities, leading them to be considered as invasive species, such the ladybird *Harmonia axyridis* (Pallas, 1773) (Coleoptera: Coccinellidae) (Roy et al., 2006; Roy & Wajnberg, 2008), the leafhopper assassin bug *Zelus renardii* Kolenati, 1857 (Hemiptera: Reduviidae) (Rosenheim et al., 1999), and the thistle head weevil *Rhinocyllus conicus* (Frölich, 1792) (Coleoptera: Curculionidae) (Louda et al., 1997). An undesired effect of the introduction of these species is their attack on non-target native species, sometimes even other beneficial species used as biological controls in agricultural systems (e.g., Rose et al., 2005; Louda et al., 1997). Fortunately, and taking a broad historical perspective, classical biological control as a scientific discipline continues to evolve and has undergone paradigm shifts, improving and optimising itself to avoid and overcome these problems (Heimpel & Cock, 2018). Among these improvements, the potential impacts of introducing an exotic biological control agent must be properly evaluated in any biological control strategy (van Driesche & Reardon, 2004).

Here, we studied the parasitoid wasp *Torymus sinensis* Kamijo, 1982 (Hymenoptera: Chalcidoidea: Torymidae), which was introduced in Europe (Italy) from Japan (even if it is native to China) in 2005 (Quacchia et al., 2008) to control the invasive Asian chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu, 1951 (Hymenoptera: Cynipidae). *Dryocosmus kuriphilus* is considered the most detrimental insect pest affecting *Castanea* trees in Asia, North America, and Europe (Oho & Umeya, 1975; Payne et al., 1975; Brussino et al., 2002), coming to cause yield reductions of 50–75% in the USA (Payne et al., 1983). This wasp, also native to China, infests chestnut orchards and forests, causing a progressive loss of the photosynthetic biomass. High gall densities on these trees also cause a reduction in chestnut fruit and wood production and sometimes leave the trees severely damaged or weakened (EFSA, 2010; Gehring et al., 2018; 2019). The high invasiveness and strong effects of this pest are likely due to the lack of natural enemies in the invaded regions (Aebi et al., 2007; Quacchia et al., 2013; Gil-Tapetado et al., 2021a), and to its efficient reproduction as a parthenogenetic and r-strategist species (EFSA, 2010).

Currently, the only effective way of reducing the impact of

*D. kuriphilus* in invaded areas is by biological control using its natural enemy, *T. sinensis* (Otake et al., 1984; Quacchia et al., 2008; Gibbs et al., 2011). This method is effective due to their synchronized life cycle (Aebi et al., 2007; Quacchia et al., 2013; Nieves-Aldrey et al., 2019; Gil-Tapetado et al., 2021a). Like all introduced species, *T. sinensis* may negatively impact the native fauna (Gibbs et al., 2011) by, for instance, attacking native cynipids (Ferracini et al., 2015a; 2017), hybridizing with native *Torymus* species (Yara et al., 2010; Pogolotti et al., 2019), or competing with other (Ferracini et al., 2018) native parasitoids. In addition, the establishment of *T. sinensis* seems to vary in the different areas in which it has been applied as a biological control agent (Nieves-Aldrey et al., 2019), modulating the degree of impact on the native fauna (the larger the establishment, the larger the impact). *Torymus sinensis* was introduced in Spain through different biological control programs of *D. kuriphilus* since 2015 (Nieves-Aldrey et al., 2019), and the impact of this parasitoid on non-target hosts and its potential to hybridize with other *Torymus* species have not been evaluated.

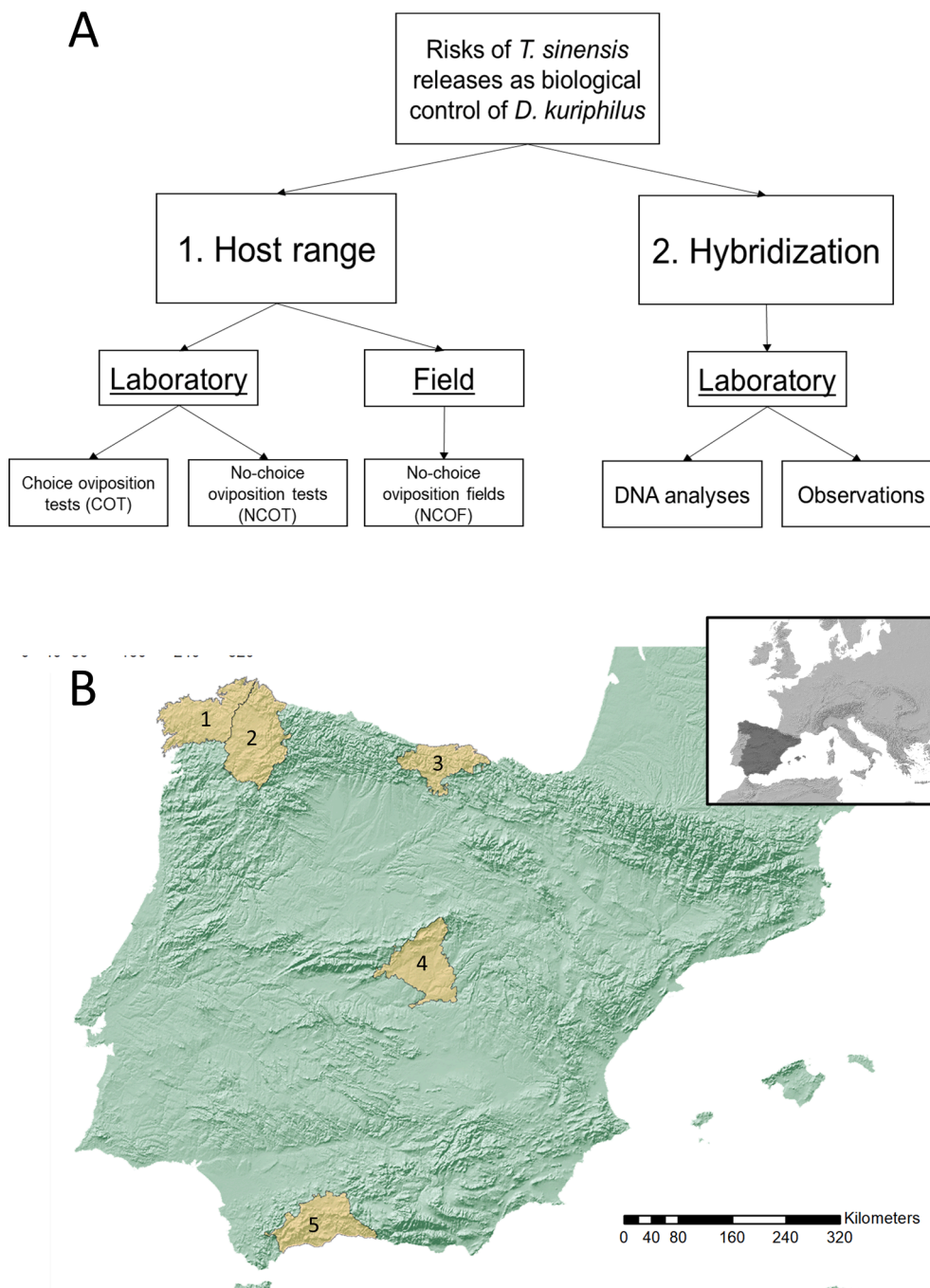
To evaluate the potential risks associated with the introduction of *T. sinensis* on native Iberian species, we first identified its host range under laboratory and field conditions in the region, focusing on the fundamental or physiological host range (i.e.: the sum of all hosts in which a species can complete its life cycle, regardless of what may occur under natural conditions, Onstad & McManus, 1996; Haye et al., 2005). We performed observational experiments in the field and/or laboratory in which mated females of *T. sinensis* were exposed to galls of non-target (native) hosts under no-choice conditions and to these galls and those of *D. kuriphilus* under choice conditions. We then performed mating experiments and a molecular phylogenetic analysis to evaluate the hybridization potential of *T. sinensis* with native *Torymus* species. For the molecular analysis, we analysed the extent of discordance between the nuclear and mitochondrial markers of these species as an indicator of genetic introgression and hybridization.

## 2. Material and methods

To examine the potential non-target risks associated with the release of *T. sinensis* in Spain with respect to physiological host range and hybridization potential, we followed the work scheme shown in Fig. 1A. To assess the risk of *T. sinensis* using native cynipid galls as hosts, we tested the physiological host range of *T. sinensis* with no choice oviposition tests in the field and with both no choice and choice oviposition tests in the laboratory. To assess the risk of *T. sinensis* hybridizing with European native species of *Torymus*, we searched for evidence of hybridization and introgression by phylogenetic analysis of selected nuclear and mitochondrial genes. We also observed, under laboratory conditions (~22 °C and with humidity levels between 30% and 50%), sexual interactions between the alien and native parasitoids. The specimens of *T. sinensis* (females) used in all the field and laboratory experiments were provided by Agrobío© and imported from Italy under dark and cold (-10 °C) conditions. The specimens of *T. sinensis* used for the molecular analyses were collected in the field.

### 2.1. Study area

Field sampling and field experiments were carried out from 2016 to 2018 in different areas of Spain: in the Northwest (Galicia: Lugo: Campus Terra, Santiago de Compostela University), the North (Cantabria: San Roque de Riomiera), the Central region (Madrid: in the Sierra de Guadarrama and the Real Jardín Botánico Alfonso XIII of the Complutense University, UCM), and the South (Andalucía: Málaga, Valle del Genal). All these areas are known to be infested by *D. kuriphilus* (Gil-Tapetado et al., 2018). *Torymus sinensis* is present in all these areas due to its release as a biological control against *D. kuriphilus* (Nieves-Aldrey et al., 2019) (Fig. 1B).



**Fig. 1.** A. Workflow of experiments carried out in this study. To evaluate the risks of *T. sinensis* releases in Spain, we focused on the (1) host range and (2) hybridization potential of the species. To evaluate the physiological host range of *T. sinensis* (1), we performed both lab and field-based oviposition experiments with galls of the selected native cynipids species. Choice oviposition tests (COT) and no choice oviposition tests (NCOT) were both conducted under laboratory conditions; in the field, only no choice oviposition (field) (NCOF) experiments were carried out. To evaluate the risk of hybridization of *T. sinensis* with other native species of *Torymus* (2), we performed both DNA analyses with *ITS2* and *cox1* and mating observations. B. Map showing the sampling areas of both the *Torymus* and the cynipid species used in the various experiments. 1) A Coruña and 2) Lugo (both in Galicia), 3) Cantabria, 4) Madrid, and 5) Málaga (in Andalucía).

## 2.2. Field sampling and collection of native cynipid galls

To evaluate the physiological host range of *T. sinensis* (♀), native cynipid galls were collected in the Sierra de Guadarrama (Madrid) in the spring to summer of 2017 and 2018. Species were selected because they share one or more of the following characteristics with *D. kuriphilus*: morphological similarity of galls (see Gil-Tapetado et al., 2021c), phylogenetic closeness (see Stone et al., 2009), ecological overlap (see Nieves-Aldrey, 2001; Gil-Tapetado et al., 2018). The galls were collected mainly from trees of *Quercus pyrenaica* Willd., *Q. ilex* L., and *Q. suber* L. These sampled galls were in good condition and at early to intermediate stages of maturity, which is when oviposition by *T. sinensis* occurs (Table 1). They were brought to the lab and used in the host range experiments. The galls induced by *D. kuriphilus* were collected from sites in

Cantabria, Madrid, and Lugo (Gil-Tapetado et al., 2018; 2021b) during the same time sampling period (Table 1). A portion of both native and alien galls were used in the laboratory experiments; the rest were bagged and used in the field experiments (Fig. 2A, B). Prior to isolating and challenging the females of *T. sinensis* in the host range experiments, all specimens of *T. sinensis* are of a similar age (parasitoids are actively collected, shipped and transported cold, so their life activity is slowed down, preventing reproductive processes until subjected to laboratory conditions) and were kept together under laboratory conditions for two days to ensure that mating was established.

## 2.3. Host range of *Torymus sinensis* in field experiments

No choice oviposition field (NCOF) experiments were conducted to

**Table 1**

Galls of cynipid species used in the field and/or laboratory experiments. The species name, tribe, gall generation type, shared characteristic with galls of *D. kuriphilus* and number of galls collected per species is provided.

Species	Tribe	Generation type	Morphological similarity	Phylogenetic closeness	Ecological overlap	N galls
<i>Dryocosmus kuriphilus</i> Yasumatsu, 1951	Cynipini	Asexual	–	–	–	237
<i>Neuroterus quercusbaccarum</i> (Linnaeus, 1758)	Cynipini	Sexual	x		x	164
<i>Plagiotrochus quercusilicis</i> (Fabricius, 1798)	Cynipini	Sexual	x	x		150
<i>Neuroterus tricolor</i> (Hartig, 1841)	Cynipini	Sexual	x		x	40
<i>Biorhiza pallida</i> (Olivier, 1791)	Cynipini	Sexual	x		x	34
<i>Andricus curvator</i> Hartig 1840	Cynipini	Sexual	x		x	33
<i>Plagiotrochus australis</i> (Mayr, 1882)	Cynipini	Sexual	x	x		24
<i>Andricus quercusramuli</i> (Linnaeus, 1761)	Cynipini	Sexual			x	15
<i>Pseudoneuroterus saliens</i> (Kollar, 1857)	Cynipini	Sexual			x	11
<i>Andricus grossulariae</i> Giraud, 1859	Cynipini	Sexual	x		x	9
<i>Synophrus hispanicus</i> Pujade-Villar, 2009	Synergini	Sexual	x			5
<i>Neuroterus politus</i> Hartig, 1840	Cynipini	Sexual	x		x	5
<i>Trigonaspis</i> sp.	Cynipini	Sexual	x			4

assess the physiological host range of *T. sinensis* in the region of Madrid. For these experiments, tree branches infested with either target (alien) or non-target (native) galls were bagged with a semipermeable mesh bag. For the experiments with native galls, the branches of several native oaks (*Quercus* spp.) were bagged; for those with galls of *D. kuriphilus*, the branches of the chestnut tree *Castanea sativa* were bagged. The mesh size chosen allowed air and water to enter but prevented the entry and exit of other small insects, avoiding possible interference by other parasitoid species in the experiment and the dispersal of *T. sinensis* in the environment. For each bagged branch, 40 specimens of *T. sinensis* (25♀/15♂) were released and, after a three-month period, the galls were collected. We assessed the presence of larvae or adults of *T. sinensis* in the galls of five native cynipid species that have either a simple or complex external morphology: *Plagiotrochus australis* (Mayr, 1882) on *Q. ilex*; *Andricus curvator* Hartig, 1840 and *Biorhiza pallida* (Olivier, 1791) on *Q. pyrenaica*; *Andricus quercusramuli* (Linnaeus, 1761) on *Q. faginea*; and *Pseudoneuroterus saliens* (Kollar, 1857) on *Q. suber*. The last two cynipids were selected to determine the interest of *T. sinensis* for galls that are morphologically different from those of *D. kuriphilus*. Four replicates were performed for each cynipid species. The bagged specimens of *T. sinensis* were kept and used for the lab-based experiments.

#### 2.4. Ex situ host range experiments

Based on approaches described by Ferracini et al. (2015a), two types of laboratory-based experiments were performed to identify the physiological host range of *T. sinensis*: 1) choice oviposition tests (COT), in which *T. sinensis* is presented simultaneously with both target and non-target galls and 2) no choice oviposition tests (NCOT), in which the species is presented with only galls of the target or one of the non-target species.

For the COT, three mated females of *T. sinensis* were exposed to galls of their main host, *D. kuriphilus*, together with non-target galls for 30 min in a 10 cm-wide Petri dish fitted with a white background and observed under a binocular stereomicroscope (Fig. 2C). During that time, the following behaviours were recorded: 1) attraction to the gall (the female walked towards the gall and onto its surface) and vibrotaxis or antennal drumming (the female made antennal movements along the gall surface), 2) attempted oviposition (the female inserted her ovipositor for less than one minute, and we consider this as a failed oviposition), and 3) successful oviposition (the female inserted her ovipositor for at least one minute, though it is unknown whether she laid eggs). Twenty replicates of this experiment were performed with the species *Biorhiza pallida*, *Neuroterus quercusbaccarum* (Linnaeus, 1758), *Plagiotrochus australis*, and *P. quercusilicis* (Fabricius, 1798) as their galls were among the most abundant collected. Data from the COT with sufficient replicates were analysed statistically using a  $\chi^2$  test. We tested whether there were significant differences in preference (i.e., number of behaviours observed towards different galls) towards the galls of *D. kuriphilus*

versus those of the non-target species.

For the NCOT, three mated females of *T. sinensis* were placed under the same conditions as in the COT, but in this case, they were presented with only the galls of the following cynipids: *A. curvator*, *Pl. australis*, *B. pallida*, *D. kuriphilus*, *Pl. quercusilicis*, *N. quercusbaccarum*, *A. quercusramuli*, *Andricus grossulariae* Giraud, 1859, *Neuroterus tricolor* (Hartig, 1841), *Ps. saliens*, and *Synophrus hispanicus* Pujade-Villar, 2009. The same behaviours as in the COT were recorded except for antennal drumming. In addition, to quantify the extent to which *T. sinensis* is attracted to the galls of a specific species, we evaluated the following variables: the number of both attempted (OvA) and successful (+Ov) ovipositions, the ratio of successful ovipositions to the total number of ovipositions, as a percentage (+Ov %), and overall attraction (ATT) to a potential host species as the ratio of the total number of ovipositions to the total number of galls tested per species (as percentage).

#### 2.5. Hybridization between *T. sinensis* and other *Torymus*

For the molecular phylogenetic analysis, we included the sequences of *Torymus* and outgroups used in a recent study by Gil-Tapetado et al. (2021c), the sequences of *T. sinensis* of Yara, 2006; Yara et al., 2010; Montagna et al., 2019; Viviani et al., 2019; Viciriuc et al., 2021, 12 new specimens of *T. sinensis* collected in Spain for this study, and those of *Torymus beneficus* Yasumatsu and Kamijo, 1979 retrieved from GenBank (Yara, 2006; Yara et al., 2010). The sequences of an additional, initially unidentified specimen of *T. beneficus* collected in Spain were also included in the analysis (Table 2). DNA extractions, amplification, and sequencing were as previously described in Gil-Tapetado et al. (2021c).

A phylogenetic analysis was performed to identify hybridization signals between Asian *Torymus* (*T. sinensis* and *T. beneficus*) and European *Torymus* (*Torymus cyaneus* Walker, 1847, *Torymus notatus* (Walker, 1833), *Torymus affinis* (Fonscolombe, 1832), *Torymus auratus*, *Torymus nobilis*, *Torymus geranii*, *Torymus bedeguaris*, *Torymus rubi*, and *Torymus flavipes*). Two genes were selected for sequencing: nuclear internal transcribed spacer 2 (*ITS2*) and mitochondrial cytochrome c oxidase subunit I (*coxI*). The contigs were assembled and edited using Sequencher 5.4.6 (Gene Codes Corporation, 2016), and a matrix for each gene was built. The *ITS2* matrix was aligned using MAFFT (Katoh & Standley, 2013; Mafft, 2018); the *coxI* matrix, after being translated into amino acids, was aligned manually with Mesquite (Maddison & Maddison, 2018). Bayesian inference analysis was conducted using Mr. Bayes v3.2.6 (Ronquist et al., 2012) on the CIPRES Science Gateway (<https://www.phylo.org>) (Miller et al., 2010). For both matrices, JModelTest (Darriba et al., 2012) was used to determine the best-fit nucleotide substitution model for our data. We performed two independent runs of 100 million generations, sampling every 1000, with four chains per run and a burn-in factor of 0.25. The nucleotide substitution model that best fit both genes was GTR + I + G. The resulting trees were visualized with FigTree 1.4.3 (Rambaut, 2014) and edited with Inkscape



**Fig. 2.** *Torymus sinensis* on target and non-target cynipid galls in the field and lab experiments. (A, B) Images showing the branch bagging set-up in which *T. sinensis* is released on chestnut branches infested with the invasive species *D. kuriphilus*; an example of a no choice oviposition test (NCOT) under laboratory conditions with galls of *Neuroterus tricolor* (C); females of *T. sinensis* ovipositing on galls of *D. kuriphilus* in a COT or NCOT (D–F); females of *T. sinensis* ovipositing on oak galls of the sexual generations of *Plagiotrochus quercuslicis* (G), *Biorhiza pallida* (H), *Neuroterus quercusbaccarum* (I), and *Trigonaspis* sp. (J) in a COT or NCOT. Photographs © J. L. Nieves-Aldrey, except (J) © D. Gil-Tapetado.

and Adobe Photoshop CC. We showed only the topologies of the subclades closest to *T. sinensis* to focus on possible hybridizations between the species of interest (the complete phylogenetic study of *Torymus* parasitoids of the Cynipidae can be found in Gil-Tapetado et al., 2021c). A BLASTN analysis (Zhang et al., 2000) was performed to compare the Tor\_ben139\_65 sequence with those in GenBank to verify the specimen's species identification as *T. beneficus*.

Additionally, observational mating experiments were performed between males and females of *T. sinensis* and the phylogenetically close native Iberian species *T. flavipes*, *T. affinis*, *T. cyaneus*, and *T. notatus*. Mating experiments were performed for 30 min and the following four behaviours were recorded: antennal contact, courtship, mating attempt,

and mating (based on Ferracini et al., 2017).

### 3. Results

#### 3.1. Host range in field experiments

In 2017, we conducted 35 NCOF experiments with three cynipid species: *B. pallida* and *A. curvator* on *Q. pyrenaica*, and *Pl. australis* on *Q. ilex*. The results were negative in all cases: no larvae or adults of *T. sinensis* were recovered from the galls of these potential hosts. In May of 2018, we conducted another 65 NCOF experiments with the galls of 5 different cynipid gall-inducing species (the same as in 2017 plus

**Table 2** Specimen voucher information and GenBank accession numbers of the new sequences of *T. sinensis* and *T. beneficus* analysed in this work. Information on the other sequences included in the phylogenetic analysis can be found in Gil-Tapetado et al. (2021c).

Species	Host	Host Plant	Emergence date	Location	Lat.	Long.	Voucher	GenBank Accession Number			Collection ID Number (MNCN/ADN)
								Folmer (cox1)	Jerry&Pat (cox1)	ITS2	
<i>Torymus sinensis</i>	<i>D. kuriphilus</i>	<i>C. sativa</i>	05/2016	Italy	43.01	12.51	Tor_sin110_52	QQ318603	-	QQ320043	119,282
"	"	"	05/2016	"	"	"	Tor_sin113_52	QQ318604	QQ318612	QQ320044	119,284
"	"	"	01/2018	Spain, Barcelona	41.803	2.421	Tor_sin114_53	QQ318605	QQ318613	QQ320045	119,285
"	"	"	01/2018	Spain, Madrid	40.447	-3.726	Tor_sin117_54	-	-	QQ320046	119,286
"	"	"	01/2018	"	"	"	Tor_sin118_54	-	QQ318614	QQ320047	119,287
"	"	"	01/2018	"	"	"	Tor_sin119_54	-	-	QQ320048	119,288
"	"	"	03/2018	Spain, Navarra	43.281	-1.683	Tor_sin130_60	QQ318606	QQ318615	QQ320049	119,229
"	"	"	03/2018	Spain, Guipúzcoa	43.367	-1.821	Tor_sin132_61	-	-	QQ320050	119,289
"	"	"	03/2018	"	"	"	Tor_sin133_61	QQ318607	QQ318616	QQ320051	119,290
"	"	"	03/2018	"	43.323	-1.883	Tor_sin135_62	QQ318608	QQ318617	QQ320052	119,291
"	"	"	03/2018	"	43.365	-1.804	Tor_sin136_63	QQ318609	QQ318618	QQ320053	119,292
"	"	"	01/2018	Spain, Málaga	36.635	-5.121	Tor_sin138_64	QQ318610	QQ318619	-	119,293
<i>Torymus beneficus</i>	<i>D. kuriphilus</i>	<i>C. sativa</i>	01/2018	"	36.626	-5.172	Tor_ben139_65	QQ318611	QQ318620	-	119,294

*A. quercusramuli* on *Q. faginea*; and *Ps. saliens* on *Q. suber*) from 13 sites across Madrid, Cantabria, and Andalucía. We did not observe parasitism of *T. sinensis* in any of these experiments, except in the galls of the sexual generation of *A. curvator*, from which we recovered five males. This fact confirms that *T. sinensis* can successfully oviposit in native cynipid oak galls in the field. The attraction of *T. sinensis* for the galls of *A. curvator* was also confirmed in the COT and NCOT experiments (see below).

### 3.2. Ex situ host range experiments

To further explore the physiological host range of *T. sinensis*, we performed, under laboratory conditions, a total of 44 oviposition tests with the galls of 11 species of Cynipidae. Evidence of attraction and ovipositor insertion was observed under laboratory conditions for all the galls except those of *A. quercusramuli*, *Ps. saliens*, and *S. hispanicus*.

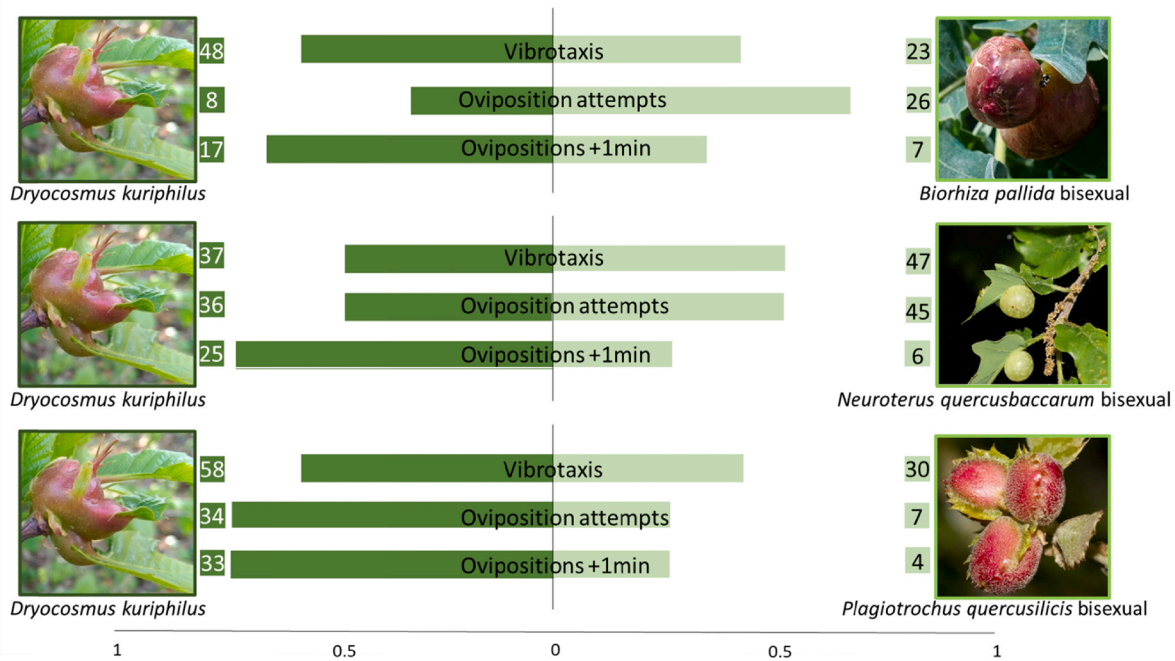
In the COT, we found that *T. sinensis* preferentially parasitized galls of *D. kuriphilus* but can also parasitize non-target galls (Fig. 3). Significant differences in the vibrotaxis behaviour of *T. sinensis* were observed: the species displayed more vibrotaxis on the galls of *D. kuriphilus* than those of *B. pallida* ( $\chi^2 = 8.23$ ,  $df = 1$ ,  $p = 0.004$ ) and *Pl. quercusilicis* ( $\chi^2 = 8.9$ ,  $df = 1$ ,  $p = 0.02$ ), however, no significant difference was observed between *D. kuriphilus* and *N. quercusbaccarum* ( $\chi^2 = 1.19$ ,  $df = 1$ ,  $p = 0.27$ ). Likewise, significant differences in the number of oviposition attempts were observed between *D. kuriphilus* and the native species *B. pallida* ( $\chi^2 = 9.53$ ,  $df = 1$ ,  $p = 0.002$ ) and *Pl. quercusilicis* ( $\chi^2 = 17.78$ ,  $df = 1$ ,  $p < 0.0001$ ). In the case of *B. pallida*, there are significantly more attempts against this species than against *D. kuriphilus*, and in the case of *Pl. quercusbaccarum* there are more attempts against the alien cynipid than against the native one. No difference in oviposition attempts was found between *N. quercusbaccarum* and *D. kuriphilus* ( $\chi^2 = 1$ ,  $df = 1$ ,  $p = 0.32$ ). For insertions lasting longer than one minute (a proxy of successful ovipositions), the preference for *D. kuriphilus* was significantly larger than that for *Pl. quercusilicis* ( $\chi^2 = 22.73$ ,  $df = 1$ ,  $p < 0.0001$ ) or *N. quercusbaccarum* ( $\chi^2 = 11.64$ ,  $df = 1$ ,  $p < 0.0001$ ); however, no significant preference difference was found between *B. pallida* and *D. kuriphilus* ( $\chi^2 = 2.27$ ;  $df = 1$ ,  $p = 0.13$ ) (Fig. 3).

Unfortunately, we did not have enough samples to perform a rigorous statistical analysis of the preference of *T. sinensis* for the galls of the other cynipid species included in the study (see Table 1). However, based on qualitative observations, we observed that *T. sinensis* did not show any attraction or ovipositing behaviours towards the galls of *N. politus*, *Ps. saliens*, or *S. hispanicus* when those of *D. kuriphilus* were present. We observed some attraction/vibrotaxis behaviours towards the filamentous catkin galls of *A. quercusramuli*, but not oviposition. In contrast, we observed ovipositioning of *T. sinensis* on the galls of *A. curvator*, consistent with the field experiments, and on the semi-subterranean galls found on stolons induced by the sexual generation of *Trigonaspis* sp. (Fig. 2J).

In the NCOT, the variables of attraction (ATT) and the ratio of successful ovipositions to the total number of ovipositions (+Ov%) were highest for the galls of *A. curvator*, followed by *Pl. australis* and *B. pallida*. The galls of *D. kuriphilus* received a similar degree of attraction and insertions as the aforementioned species. Less attractive galls were those induced by *A. grossulariae*, *Pl. quercusilicis*, *N. quercusbaccarum*, and *N. tricolor*, with the least attractive ones (i.e., no ovipositions) being those induced by *A. quercusramuli*, *Ps. saliens*, and *S. hispanicus* (Table 3).

### 3.3. Phylogeny, hybridization, and mating experiments

Phylogenetic hypotheses based on *ITS2* and *cox1* data showed different relationships among closely related species and revealed episodes of hybridization between *T. beneficus* and *T. sinensis* (Fig. 4), as also observed in previous studies (see Discussion). In the *ITS2* phylogeny (Fig. 4A), most sequences of *T. sinensis* and all those of *T. beneficus* group within the same subclade with no indication of nuclear differences between the two species. However, in the *cox1* phylogeny *T. beneficus* and



**Fig. 3.** Differences in the behaviour of *T. sinensis* in the choice oviposition tests (COT). The three behaviours considered were vibrotaxis, oviposition attempts, and oviposition lasting longer than one minute. Females of *T. sinensis* were exposed to galls of *D. kuriphilus* (target) and the following non-target, native species of Cynipidae: *Plagiotrochus quercusilicis*, *Neuroterus quercusbaccarum*, and *Biorhiza pallida* (all bisexual generations). Dark green bars indicate the predilection of *T. sinensis* to the galls of *D. kuriphilus* and light green bars, to those of the native species. Numbers in the green squares indicate the total number of each behaviour of *T. sinensis*. Significance of the Chi-square results are also shown (\*<0.05; \*\*<0.01; \*\*\*<0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 3**

Results of the no choice oviposition tests with *T. sinensis* against *D. kuriphilus* (target species) and the native cynipid gall species (non-target). Generation type is indicated in parentheses (asex: parthenogenetic; sex: bisexual). +Ov % = proportion of successful ovipositions (+Ov N) over total ovipositions (failed (OvA N) + successful (+Ov N)). ATT = ratio between +Ov N and the number of galls used in the experiments (as percentage). The species are ordered by decreasing ATT.

Species	N galls	OvA N	+Ov N	+Ov %	ATT
<i>Andricus curvator</i> (sex)	27	7	53	88.3	222.2
<i>Plagiotrochus australis</i> (sex)	24	16	22	57.9	158.3
<i>Biorhiza pallida</i> (sex)	6	4	5	55.6	150
<i>Dryocosmus kuriphilus</i> (asex)	8	4	4	50	100
<i>Andricus grossulariae</i> (sex)	9	7	1	12.5	88.9
<i>Plagiotrochus quercusilicis</i> (sex)	55	11	15	57.7	47.3
<i>Neuroterus quercusbaccarum</i> (sex)	67	15	6	28.6	31.3
<i>Neuroterus tricolor</i> (sex)	40	0	3	100	7.5
<i>Andricus quercusramuli</i> (sex)	10	0	0	0	0
<i>Pseudoneuroterus saliens</i> (sex)	11	0	0	0	0
<i>Synophrus hispanicus</i> (sex)	5	0	0	0	0

*T. sinensis* form a sister group (with four *T. beneficus* sequences in the *sinensis* clade) and this clade is the sister group to *T. notatus* (Fig. 4B). In this case, *T. beneficus* forms a subclade that is largely distinct from that of *T. sinensis*, with a few sequences of *T. beneficus* clustered within the *T. sinensis* clade, indicative of hybridization.

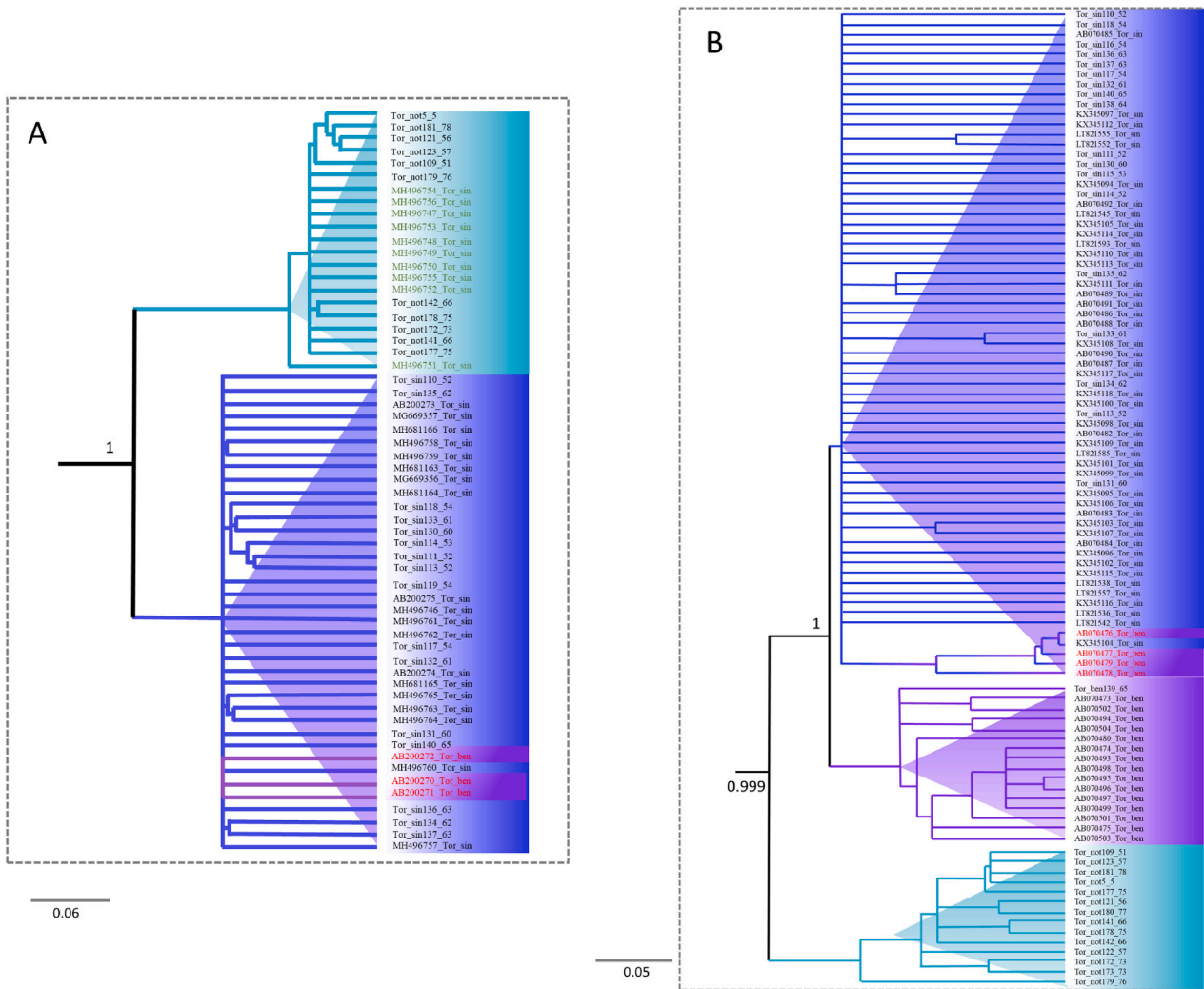
In the ITS2 analysis, some sequences labelled as *T. sinensis* resolved within the subclade comprising the sequences of *T. notatus* (Fig. 4A), however, this situation is likely due to cases of misidentification and not hybridization (see Discussion). Also, the mitochondrial sequence of a morphologically unidentified specimen of *Torymus* (Tor\_ben139\_65) that was collected in Júzcar (southern Spain) nested within the subclade of *T. beneficus*, representing the first record of this Japanese species in Europe.

No mating attempts between *T. sinensis* and the native Iberian species *T. flavipes*, *T. affinis*, *T. cyaneus* and *T. notatus* were observed under laboratory conditions (Table 4). However, a few instances of courtship and antennal contact between *T. sinensis* and *T. notatus* and *T. affinis* were observed. These behaviours, always initiated by the males, were ignored by the females. In the trials between males and females of *T. sinensis*, all four attraction and copulation behaviours were observed, as expected.

#### 4. Discussion

##### 4.1. Host range in field experiments

The detection of *T. sinensis* emerging from galls of *A. curvator* confirms that this exotic species can parasitize native Iberian species and that there is a potential for it to become part of oak gall communities in the future. A high density of *T. sinensis* in the environment, together with a broad physiological host range, could cause damage to the native biological communities of oak galls (Ferracini et al. 2015a; 2017). Also, this finding highlights another concern: the possible effects of competition exerted by *T. sinensis* towards native parasitoids when attacking non-target galls. Ferracini et al. (2015a) reported the emergence of three male *T. sinensis* from field-collected galls of *B. pallida* and observed female *T. sinensis* laying eggs on the galls of *A. curvator* under laboratory conditions. Host range expansion of *T. sinensis* in Italy was also later confirmed by Ferracini et al. (2017). Our results show limited attack by *T. sinensis* even under artificial field conditions designed for maximum exposure of non-target hosts. None of the non-targets were attacked, except *A. curvator*, and even then, the offspring were males, so in the observed case they could not parasitise the hosts. However, it is possible that over the years and considering the large number of *T. sinensis* in the environment, such attacks on non-target galls may occur more frequently. Only monitoring of native Cynipidae galls in the field can provide information on whether there is a real risk that the host range is



**Fig. 4.** A. Phylogram based on *ITS2* focusing on the subclade comprising *T. notatus*, *T. sinensis*, and *T. beneficus*. Sequences marked in red indicate those of *T. beneficus* that cluster within the *T. sinensis* clade. Sequences marked in green indicate those (mis)identified as *T. sinensis* that group within the *T. notatus* clade (see Discussion). B. Phylogram based on *cox1* focusing on the subclade comprising *T. notatus*, *T. sinensis*, and *T. beneficus*. Sequences marked in red indicate those of *T. beneficus* that group within the *T. sinensis* clade. Values on nodes mark the posterior probability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 4**

Results of the hybridization trials among *T. sinensis* and native species of *Torymus* (*T. flavipes*, *T. affinis*, *T. cyaneus*, and *T. notatus*). The table shows the number of trials (which depended on specimen availability) for each species combination and the number of movements detected for four behaviours: antennal contact, courtship (when the *Torymus* male intercepts the female and performs movements related to a “courtship dance”), mating attempt (rapid and incomplete copulation behaviour), and mating (copulation lasting several minutes). Trials were also conducted between males and females of *T. sinensis* to obtain a blank experiment with which to compare the rest of the experiments.

Number of trials	Hybridization trial		Antennal contact	Courtship	Mating attempt	Mating
4	<i>T. sinensis</i> ♂	x <i>T. sinensis</i> ♀	4	4	2	3
4	<i>T. sinensis</i> ♂	x <i>T. cyaneus</i> ♀	0	0	0	0
1	<i>T. cyaneus</i> ♂	x <i>T. sinensis</i> ♀	1	0	0	0
2	<i>T. sinensis</i> ♂	x <i>T. flavipes</i> ♀	0	0	0	0
1	<i>T. flavipes</i> ♂	x <i>T. sinensis</i> ♀	0	0	0	0
4	<i>T. notatus</i> ♂	x <i>T. sinensis</i> ♀	1	1	0	0
5	<i>T. sinensis</i> ♂	x <i>T. notatus</i> ♀	1	0	0	0
9	<i>T. sinensis</i> ♂	x <i>T. affinis</i> ♀	1	1	0	0
9	<i>T. affinis</i> ♂	x <i>T. sinensis</i> ♀	0	0	0	0

wide and may negatively affect native species.

While there is no doubt that the control exerted by *T. sinensis* against *D. kuriphilus* is efficient, we question the specificity of the species to *D. kuriphilus*. We do not know if its mechanism of control is directed against only the invasive cynipid or those species with a simple gall

morphology (e.g., *D. kuriphilus*, *A. curvator*, and *B. pallida*). The election of *T. sinensis* to control populations of *D. kuriphilus* outside China is based on this parasitoid species having the largest abundance in the biological communities containing *D. kuriphilus* in China, thereby having the greatest effect on the population regulation of this cynipid. However,



parasitoid and cynipid communities in China (i.e., the native range of *D. kuriphilus* and *T. sinensis*) have not yet been studied, and it is possible that *T. sinensis* is not the most specialist and effective natural enemy against *D. kuriphilus*. Taking a broader view, it is possible that *T. sinensis* is simply the most abundant parasitoid in the cynipid communities in China and a more polyphagous species than described.

#### 4.2. Ex situ host range experiments

In accordance with Ferracini et al. (2015a), the observed ovipositions of *T. sinensis* in native galls in the COT and NCOT (Fig. 3 and Table 3) highlight that this parasitoid is not monophagous or a specialist of *D. kuriphilus* but rather has a wider physiological host range than expected. *Torymus sinensis* appears to be attracted to even underground tree stratum galls such as those induced by sexual generations of *Trigonaspis* sp. (see Fig. 2J). Considering the gall morphology of the native species of Cynipidae used in our experiments, *T. sinensis* seems to be particularly attracted to globular and simple galls, such as those of *D. kuriphilus*, *Pl. quercusilicis*, *A. curvator*, or *N. quercusbaccarum* (sexual generation) (see Fig. 2). Indeed, the species with the highest level of parasitism by *T. sinensis* in the NCOT was *A. curvator*, which was also the only non-target species parasitized in the field experiments, suggesting it could be one of the most vulnerable to *T. sinensis*. Also, the connection between *T. sinensis* and its potential parasitism of native Cynipini galls with a morphology shared by or similar to that of *D. kuriphilus*, which may be related to a parasitism niche, fits with the ‘ecological fitting’ hypothesis (Agosta et al., 2010) and the ‘Stockholm paradigm’ (Brooks et al., 2019).

As previously mentioned, *T. sinensis* is known to be an effective biological control agent against *D. kuriphilus* in different countries (e.g., Borowiec et al., 2014; 2018; Ferracini et al., 2015b, 2019), although its efficacy appears to vary by region (Nieves-Aldrey et al., 2019). For instance, in Spain, a pest decline was reported in the border area with France in the regions of Navarra (Nieves-Aldrey et al., 2019) and Catalonia (Managers of the Natural Park of the Zona Volcànica de la Garrotxa and the Natural Park of Montseny, personal communication), likely due to the natural dispersion of the parasitoid from France. In other Spanish territories, however, *T. sinensis* has not been as highly effective (Nieves-Aldrey et al., 2019), although its establishment and effect on *D. kuriphilus* is expected to increase in these regions in the future. Here, we focus especially on the physiological host range of the species, but the realized or ecological host range (the current and evolutionary set of hosts that a species actually uses to reproduce successfully in the wild, Onstad & McManus, 1996; Haye et al., 2005) should be investigated, extending this study with the monitoring of native Cynipidae communities in oak trees that share habitat with *D. kuriphilus* in chestnut trees.

#### 4.3. Hybridization of *T. sinensis*

Our results confirm that *T. sinensis* and *T. beneficus* are closely related and may represent a single evolutionary unit, as indicated by previous studies (Yara, 2006; Yara et al., 2010; Montagna et al., 2019; Viviani et al., 2019; Viciriuc et al., 2021), with the presence of hybrids (Yara et al., 2010; Viciriuc et al., 2021) that, in some studies, are considered as the same species (Viciriuc et al., 2021). In fact, only the *cox1* data set revealed differences between *T. sinensis* and *T. beneficus*, however, these data do not confirm these clades as reciprocally monophyletic but rather show evidence of introgression between them. It is possible that there was a recent geographic substructuring or incipient speciation process between *T. sinensis* on the Asian continent and *T. beneficus* isolated on the islands of Japan, with the East Sea acting as a geographical barrier. In this scenario, population-level differences indicated by mitochondrial *cox1* may reflect the influence of the past barrier, with the homogeneous nuclear data (*ITS2*) reflecting incomplete isolation. The introduction of *T. sinensis* to control *D. kuriphilus* in Japan (Otake et al., 1984) may have

halted the geographic substructuring or speciation process by allowing gene flow to resume between mainland and island populations. Further explanations of the hybridization between *T. sinensis* and *T. beneficus* are detailed in the studies of Yara et al. (2010) and Viciriuc et al. (2021). Based on the evidence to date, we concur with the hypothesis of Viciriuc et al. (2021) in which *T. sinensis* and *T. beneficus* are not independent taxa, but partially isolated populations of a single evolutionary unit.

The specimen of *T. beneficus* (Tor\_ben139\_65) collected in Spain represent the first record of this species in Europe. Its presence in Spain is probably because the biological control samples of *T. sinensis* used in Europe originated in Japan, where *T. sinensis* and *T. beneficus* share habitats and hybridize. Moreover, the high morphological similarity between the two species makes them difficult to differentiate from one another and has caused their accidental entry into the Western Palaearctic.

The sister species of *T. sinensis* plus *T. beneficus* is the European *T. notatus*, as previously shown by other phylogenetic studies (Pogolotti et al., 2019; Gil-Tapetado et al., 2021a; 2021c). *Torymus sinensis* and *T. notatus* (Western Palaearctic) are highly similar morphologically and their differentiation is usually complicated (Gil-Tapetado et al., 2021a). Hybridization between *T. notatus* and *T. sinensis* would be an *a priori* risk in biological control strategies based on *T. sinensis* (Pogolotti et al., 2019; Gil-Tapetado et al., 2021c). In our *ITS2* phylogeny, the Italian specimens of *T. sinensis* (sequences from Viviani et al., 2019) nested within the clade comprising *T. notatus* (see Fig. 4A). We believe that these specimens were misidentified as *T. sinensis* and are actually *T. notatus*. Pogolotti et al. (2019) previously confirmed the presence of *T. notatus* in Italy; however, Viviani et al. (2019) did not include *T. notatus* in their study, leading to the incorrect assignment of their specimens. Besides these likely misidentifications, we did not observe any molecular evidence of introgression between *T. sinensis* and *T. notatus* (Fig. 4).

Despite the phylogenetic closeness of *T. notatus* and *T. sinensis*, their shared morphological characters, and similar life cycles (Pogolotti et al., 2019; Gil-Tapetado et al., 2021c), the genetic divergence between these species is quite large, supporting the low probability of hybridization between them, thus minimizing this risk in the release of *T. sinensis* as a biological control agent. In addition, hybridization experiments between the two species did not yield any relevant results. Some attraction of males of *T. notatus* and *T. affinis* (the native species phylogenetically closest to the clade comprising *T. sinensis* and *T. beneficus*) to females of *T. sinensis* was observed in the mating experiments, but in all cases, the females ignored the males of these species (Table 4).

To confirm this, other more powerful molecular techniques should be carried out to study hybridization between these species, such as AFLPs or RADseq analysis (e.g.: Bangert et al., 2006; Eaton & Ree, 2013). This would further minimize the risk of introducing an alien species into the wild.

## 5. Conclusions

We question why the European *T. notatus* was not considered as an alternative control species for *D. kuriphilus*, given that it presents a similar biology, ecology, and physiological host range as *T. sinensis* (Gil-Tapetado et al., 2021a, 2021c). This conservation in traits suggests *T. notatus* could have been a more appropriate species for biological control, potentially along with the rest of the native western Palaearctic species from the overwintering subcommunity (Gil-Tapetado et al., 2021a; Kos et al., 2015). A biological control study with *T. notatus* should have been carried out earlier, but now, despite indications of its efficacy against *D. kuriphilus*, it is too late. *Torymus sinensis* has already become established in the natural environment and its elimination would be difficult. Undoubtedly, conservation or augmentation biological control with *T. notatus* would also have negatively affected susceptible native cynipid species, such as *A. curvator*, but at least the imbalance would have been made by a species already present in the European natural environment. Although our study provides some clues,

only subsequent field monitoring will indicate whether the role of *T. sinensis* as an effective biological control on *D. kuriphilus* is neutral or detrimental to the native fauna.

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#### Ethical guidelines

Ethics approval was not required for this research.

#### CRedit authorship contribution statement

**Diego Gil-Tapetado:** Conceptualization, Investigation, Methodology, Data curation, Resources, Writing – original draft. **E. Karen López-Estrada:** Investigation, Methodology, Data curation. **Yolanda Jiménez Ruiz:** Investigation, Methodology, Supervision. **Francisco José Cabrero-Sañudo:** Investigation, Methodology, Supervision, Writing – review & editing. **José F. Gómez:** Conceptualization, Supervision, Writing – review & editing. **Patricia Durán Montes:** Investigation, Data curation. **Carmen Rey del Castillo:** Resources. **M. Pilar Rodríguez-Rojo:** Resources. **Carlo Polidori:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing. **José-Luis Nieves-Aldrey:** Conceptualization, Investigation, Funding acquisition, Resources, Supervision, Writing – review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability statement

The data presented in this article is available on reasonable request from the corresponding author.

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