original research article

Biological control of invasive *Dryocosmus kuriphilus* with introduced parasitoid *Torymus sinensis* in Croatia, Slovenia and Hungary

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Abstract

Background and purpose: Dryocosmus kuriphilus *is considered as one of the major pests of sweet chestnut and the effective method of controlling its populations and damage is the biological control with its introduced parasitoid* Torymus sinensis. T. sinensis *is a univoltine, host specific parasitoid, phenologically synchronized and morphologically adapted to* D. kuriphilus. *It has a good dispersal ability, it builds up populations quickly and it effectively controls the pest already few years after the release. In this paper we describe the first results of the introduction and release of* T. sinensis *for controlling the populations of invasive* D. kuriphilus *in sweet chestnut forests and orchards in Croatia, Hungary and Slovenia.*

Materials and methods: *Withered* D. kuriphilus *galls with* T. sinensis *larvae were collected in Torino vicinities, Italy, in March 2014 and March 2015. After emergence, males and females were put together, kept at 14°C and fed with liquid honey until the release. To confirm the establishment of the population of* T. sinensis *on release site from 2014 in Croatia morphological and DNA identification were used. Fresh galls from the release site of Pazin, Croatia were collected in spring 2015 and parasitism rates of* T. sinensis *were calculated.*

Results and conclusions: *In total 10,590 females and 5,295 males of* T. sinensis *were released in sweet chestnut forests in Croatia, Hungary and Slovenia in 2014 and 2015. The minimum release number of* T. sinensis *was 100 females/50 males and the maximum was 1,200 females per site. Subsequent releases with a larger number of females (1,200 and 800 in two years) resulted in a rapid establishment of the population of* T. sinensis *and parasitism rates of 76% only one year after the first release was done. The establishment of the population of* T. sinensis *was confirmed with molecular analyses and morphological identification. Obtained haplotype sequences of* T. sinensis *have shown similarities with* Torymus beneficus. *Prior to the release of* T. sinensis*, all advantages and disadvantages of the introduction of this alien biocontrol species into natural stands in Croatia, Hungary and Slovenia have been considered.*

INTRODUCTION

Accidental introductions of alien insect species to Europe have become more and more frequent in recent decades (1, 2, 3). *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera; Cynipidae), an invasive insect pest on sweet chestnut (*Castanea* spp.) has rapidly spread throughout Europe

since the introduction to Italy in 2002 (4, 5, 6). It is considered as one of the major pests on sweet chestnut, causing yield losses up to 80% (5). In its native range in China, *D. kuriphilus* populations are kept at low, non-damaging levels by natural enemies (7, 8, 9). This is not the case in the invaded countries (Japan, South Korea, the USA, Italy, Slovenia, Croatia, Hungary and France) where the indigenous parasitoids have already shifted onto the new host but their parasitism rates are low and have little influence on the population density of their host (7, 8, 10, 11, 12, 13). *Torymus sinensis* Kamijo (Hymenoptera: Torymidae) is a parasitoid coming from China like its host and has been proven to be the only effective method of controlling the populations of *D. kuriphilus*. This specific parasitoid has been successfully introduced to Japan, the USA, Italy and France and used in classical biological control and it has reduced the populations of *D. kuriphilus* to tolerable levels (infestation less than 30%) (6, 14, 15, 16, 17, 18,). Adults of *T. sinensis* emerge from withered galls in early spring and, after mating, females lay eggs into newly formed galls, either onto the body surface of the host larva or on the wall of the larval chamber. The parasitoid larva feeds ectoparasitically on the pest's mature larva and pupates during late winter (6). *T. sinensis* is univoltine, host specific parasitoid, phenologically synchronized and morphologically adapted to *D. kuriphilus*. It has good dispersal abilities, it builds up populations quickly and it effectively controls the pest already few years after the release (6, 17). In Italy, on sites with releases of *T. sinensis* in 2005 and 2006, in 6-7 years parasitism rates have reached on average 67 % (up to 95% in some sites (17)). All these traits influence the host populations and reduce the number of galls and the damage to tolerable levels.

Studies of native parasitoids have shown that *D. kuriphilus* has recruited a number of oak gall wasp generalist parasitoids, among which are several species from the genus *Torymus* (*T. flavipes* (Walker), *T. geranii* (Walker), *T. notatus* (Walker), *T. auratus* (Müller)) (11, 12, 13, 19). However, the effectiveness of the native parasitoids is by far not enough to suppress the populations of *D. kuriphilus* and keep them under economic threshold, so the average percentage of parasitism is low, around 2 – 4.5% (12, 15)

The introduction of *T. sinensis* is one of the successful cases of biological control of highly invasive species (14, 15, 16, 17). Considering *T. sinensis* as a valid and successful management option against *D. kuriphilus*, Croatia, Hungary and Slovenia have started to introduce this parasitoid species to native sweet chestnut stands and orchards, evaluating beforehand all the advantages and disadvantages of releasing this „planned biological invasion" to natural forest stands, since *T. sinensis* is an alien species as well.

The aim of this paper is to present the first results of the introduction and release of *T. sinensis* for controlling the populations of *D. kuriphilus* in sweet chestnut forests and orchards in Croatia, Hungary and Slovenia.

MATERIAL AND METHODS

Rearing and release

The same laboratory rearing method of *T. sinensis* was used in Croatia, Slovenia and Hungary. Withered *D. kuriphilus* galls with *T. sinensis* larvae were collected on 11th March 2014 and 2nd March 2015 in Borgo d'Ale, Piemonte, Italy in a sweet chestnut orchard. More than 12,000 galls were collected. The parasitism rate of *T. sinensis* in collected galls was more than 90% (17, G. Bosio, personal communication). After the collection, the galls were stored in the refrigerator at 4°C, in glass jars with aerated tops, app. 70 galls per jar in the following entomological laboratories: Croatian Forest Research Institute, Plant Health and Molecular Biology Laboratory, National Food Chain Safety Office, Hungary, and Slovenian Agriculture and Forestry Institute Nova Gorica, Department for Plant Protection. On 1st April 2014 and 1st April 2015 the galls were taken out from the fridge and were then kept at room temperature (20-22°C, L:D 12:12) until the emergence of adults. The glass jars were divided in lots, and each lot was taken out in weekly intervals. This has provided us with a constant supply of fresh adults which could be released in the field within few days.

After the emergence, males and females were put into 50 ml plastic centrifuge tubes with plastic tops (10 females and 5 males per tube) and fed with drops of liquid honey. They were kept in a climatic chamber at 14°C (L:D 12:12) until release, with honey checked and changed every second to fourth day. All the released adults were less than a week old on the day of the release in Croatia and Slovenia and three week old females were also released in Hungary. The sweet chestnuts in the forests and orchards were checked once per week for the development of leaves and galls to determine the optimal gall size and release date for each site. Field releases (Figure 1) were done on 11th April 2014 in Croatia, 26th May 2014 in Hungary, from 16th April to 19th April 2015 in Croatia, from 28th April to 25th May 2015 in Hungary and from 22nd April to 29th April in Slovenia.

To confirm the establishment of the population of *T. sinensis* on release sites from 2014, withered galls were collected in March 2015 (localities shown in Table 1) and stored in rearing cages at room temperature (20-22°C). After a few days, parasitoid adults emerged. They were stored in alcohol (70%) and identified morphologically and by means of molecular biology, using a segment of barcode marker described by Folmer et al. (1994) (20).

DNA protocols and identification

Insect DNA was extracted from 68 specimens (Table 1) using GenEluteTM Mammalian Genomic DNA Kit (Sigma, USA) following the manufacturer's protocol. Amplification was carried out with conventional PCR in 25 μl reactions containing 2 mM of MgCl2, 100 μM of

Table 1. *Locality codes, coordinates and the number of specimens for morphological and molecular identification of Torymus sinensis on sites in Croatia, Italy and Slovenia*

Code	Locality	Coordinates (X, Y)	Specimens
69	Croatia, tunel Učka	45.314274, 14.243933	10
71	Croatia, Ozalj	45.606554, 15.464357	10
72	Croatia, Samobor	45.791143, 15.672134	10
73	Croatia,Pazin	45.233318, 13.918450	10
74	Slovenia, Vrtojba,	45.913.980, 13.617.860	10
75	Italy, Borgo d' Ale	45.367584, 8.053120	10
77	Slovenia, Ravnica, Nova Gorica	45.973611, 13.6875	8
			68

dNTPs (Fermentas, Lithuania), 0,5 µM of each primer HCO (20) and 1859F (21), 1U of Taq polymerase (Fermentas) and 2µl of template DNA. Thermocycling consisted of an initial denaturation step at 94°C (2 min), which was followed by 45 cycles at 94°C (1 min), 50°C (1 min 30 s) and 70°C (1 min 30 s) and final extension step at 70°C (10 min). All reactions contained a negative control and were checked for amplification by gel electrophoresis. Confirmed products were purified and directly sequenced with HCO at Macrogen Europe (Amsterdam, Netherlands). The resulting sequences were delivered electronically.

Chromatograms were manually verified and the sequences were aligned using ClustalW algorithm implemented in BioEdit (22). Diversity indices and test of genetic differentiation estimates were calculated using DnaSP (23). Basic local alignment search (BLAST) (24) at NCBI Gene Bank was performed, with the divergence limit for species delimitation calculated according to Meier et al. (2006) (25), using MEGA 6 (26). Haplotype determination was performed using TCS 1.21 (27, 28)

Morphological identification and parasitism rates

For the identification of adult parasitoids, an unpublished key generated by R. R. Askew (Manchester, UK) was used, which has been a basic identification tool in use for decades in the research of parasitoid communities of oak gall wasps. The catalogue of oak gall wasp parasitoids (29) is based exclusively on this key. The part of the key with *Torymus* species is based mainly on Graham et al. (1998) (30) revision work. The part of the key generated by R.R. Askew for the identification of *Torymus* species does not include *T. sinensis,* so we added the necessary diagnostic characters for the identification of *T. sinensis*. The adopted key for European *Torymus* species which attack oak gall wasps and *D. kuriphilus* is still under revision

and will be published later with important notes on *Torymus* larvae's morphology.

For parasitism rates, 357 withered *D. kuriphilus* galls were collected in February 2015 in Pazin, Croatia (release site from 2014, area 13 ha). The parasitism rate was calculated: % of *T. sinensis* parasitism = (the number of larval chambers/the number of *T. sinensis* emerged)*100. In June 2015, fresh, spring *D. kuriphilus* galls were collected from the same site and 129 galls were dissected. In dissected galls larval chambers, the number of *T. sinensis* larvae, *D. kuriphilus* larvae and pupae, and other parasitoid larvae were counted and parasitism rates were calculated (% of *T. sinensis* parasitism = (the number of larval chambers/the number of *T. sinensis*)*100. Parasitism rates were not calculated for the release localities in Slovenia and Hungary.

RESULTS

Slovenia, Croatia and Hungary have done a joint research of native parasitoids that have shifted to *D. kuriphilus* using the same methods since 2010 (unpublished data). This cooperation has led to joint efforts in using same methods for the raising and release of *T. sinensis* at the same time in all three countries.

Croatia

First males started to emerge 10 days after being placed at room temperatures and after two days the females started to emerge. The emergence stopped after 7 days. When put into the same tubes, males and females started to mate immediately. No mortality among males or females was noticed when they were kept at constant 14°C and fed with liquid honey.

The first release in Croatia was done on 11th April 2014 in Pazin in sweet chestnut forest of 13 ha (1,200 females/600 males). The adults emerging from the withered galls from this locality in early spring 2015 were confirmed as *T. sinensis* (both by DNA and morphologically), so the establishment of the population can be confirmed. The parasitism rate after the first year's release on Pazin site was 7.3 %. In 2015 additional 800 females/400 males were released on the same site. The parasitism rate after two year's releases calculated by dissection of galls was 75.58 %.

In 2015, a total of 9,500 *T. sinensis* (6,340 mated females and 3,170 males) were released across 37 sites in continental and coastal Croatia (Figure 1). The area of release did not cover all sweet chestnut stands in Croatia. The number of females/males released varied across sites and was chosen according to the size of the forests and the intensity of infestation with *D. kuriphilus*. Most of the parasitoids flew away right after the release and some ovipositions were observed in the field.
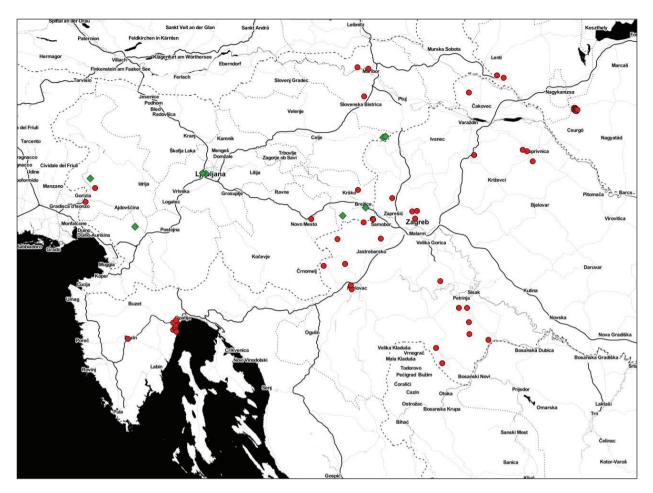
Figure 1: *Release sites of Torymus sinensis in Croatia, Hungary and Slovenia (red dots) and localities (not release sites) where T. sinensis was found in 2015 (green diamonds)*

Hungary

In Hungary the first release of *T. sinensis* was done in 2014 in three localities: Dobri, Kerkateskánd and Torny-iszentmiklós, in Zala County, the most south-western corner of Hungary, not far from the Slovenian border, on 9th April. In the first experiments isolators from fine mesh were used, a branch with 50-80 buds was enclosed by 1st April. *D. kuriphilus* galls started to form in the first week of April. In Dobri three isolators were put onto two chest-nut trees, 20 females and 10 males were released into each of isolators on 9th April. In Kerkateskánd 2 isolators were put out with the same number of *T. sinensis* females and males per isolator on the same day as in Dobri. Isolators were removed from the trees on 23rd April, after all *T. sinensis* females died. In autumn, 10 galls were collected from each branch, which were earlier covered with isola-tors, and the dissection of collected galls showed the pres-ence of *T. sinensis* larvae. In each of dissected galls at least one *T. sinensis* larvae was found. Later DNA analysis showed that the larvae belong to *T. sinensis*. On 26th May 2014 *T. sinensis* females were released onto two trees in Kerkateskánd (50 females) and Tornyiszentmiklós (200

females). In 2015, *T. sinensis* was released in Zala and Somogy counties. In Zala, 100 females in Tornyiszent-miklós and 150 females in Dobri were released. In Som-ogy County, near Iharosberény 1,950 mated females were released on 15 points (Figure 1). The parasitism rates after the release in 2014 were not calculated for Hungary.

Slovenia

The first release of *T. sinensis* in Slovenia was on 22nd April 2015 in chestnut orchard in Vrtojba. In two weeks 100 mated females and 50 males per location were re-leased on six locations in Slovenia (Figure 1).

Additionally in 2015 the collecting of overwintered galls was done in March and April in natural stands of sweet chestnut on several locations in Slovenia. Morpho-logical identification confirmed the occurrence of parasi-toid *T. sinensis* throughout the country (Baske, Orehovi-ca, Ljubljana, Kostanjevica, Čatež ob Savi, Log and Rogatec) (Figure 1 in green diamonds), although in 2014 galls were collected on the same locations and not a single specimen of *T. sinensis* was found. Genetical analyses con-

Table 2. *A selection of BLAST results at NCBI GenBank. The identity of haplotype sequences to the most similar sequences found on GenBank is displayed. Sequences within 2.7 % divergence for species delimitation are shown in bold. The first most divergent taxon is shown in grey.*

No	Species	GenBank record	Haplotypes																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*Torymus sinensis*	AB070492	**99.3**	97.7	**98.0**	**98.3**	97.7	97.7	**98.7**	97.7	97.7	97.0	**98.3**	97.3	97.3	97.3	**98.3**	97.3	**98.3**	**98.7**
2	*T. sinensis*	AB070490	**98.7**	97.7	**98.0**	**99.0**	97.7	97.7	**99.3**	97.7	97.7	97.0	**98.3**	97.3	97.3	97.3	**99.0**	97.3	**98.3**	**98.7**
3	*T. sinensis*	AB070489	**98.7**	97.7	**98.0**	**98.3**	**98.3**	**98.3**	**98.7**	**98.3**	97.7	97.0	**98.3**	97.3	97.3	97.3	**98.3**	**98.0**	97.7	**99.3**
4	*T. sinensis*	AB070488	**98.7**	97.7	**98.0**	**99.0**	97.7	97.7	**99.3**	97.7	97.7	97.0	**98.3**	97.3	97.3	97.3	**99.0**	97.3	**98.3**	**98.7**
5	*T. sinensis*	AB070491	**98.3**	97.3	97.7	**98.7**	97.3	97.3	**99.0**	97.3	97.3	96.7	**98.0**	97.0	97.0	97.0	**98.7**	97.0	**98.0**	**98.3**
6	*T. sinensis*	AB070487	**98.3**	97.3	97.7	**98.7**	**98.0**	**98.0**	**99.0**	**98.0**	97.3	96.7	**98.0**	97.0	97.0	97.0	**98.7**	97.7	**98.0**	**99.0**
7	*T. sinensis*	AB070485	**98.3**	97.3	97.7	**99.3**	97.3	97.3	**99.0**	97.3	97.3	96.7	**98.0**	97.0	97.0	97.0	**98.7**	97.0	**98.0**	**98.3**
8	*Torymus beneficus*	AB070479	**98.3**	**98.0**	**98.3**	**98.0**	**98.0**	**98.0**	**98.3**	97.7	**98.0**	97.3	**99.3**	97.7	97.7	97.7	**98.0**	97.7	97.3	**98.3**
9	*T. beneficus*	AB070478	**98.3**	**98.0**	**98.3**	**98.0**	**98.0**	**98.0**	**98.3**	97.7	**98.0**	97.3	**99.3**	97.7	97.7	97.7	**98.0**	97.7	97.3	**98.3**
10	*T. beneficus*	AB070477	**98.3**	**98.0**	**98.3**	**98.0**	**98.0**	**98.0**	**98.3**	97.7	**98.0**	97.3	**99.3**	97.7	97.7	97.7	**98.0**	97.7	97.3	**98.3**
11	*T. beneficus*	AB070476	**98.3**	**98.0**	**98.3**	**98.0**	**98.0**	**98.0**	**98.3**	97.7	**98.0**	97.3	**99.3**	97.7	97.7	97.7	**98.0**	97.7	97.3	**98.3**
12	*T. sinensis*	AB070486	**98.0**	97.0	97.3	**98.3**	97.0	97.0	**98.7**	97.0	97.0	96.3	97.7	96.6	96.7	96.7	**98.3**	96.7	97.7	**98.0**
13	*T. sinensis*	AB070484	**98.0**	**99.0**	**99.3**	97.7	**99.0**	**99.0**	**98.0**	**98.7**	**99.0**	**98.3**	**98.3**	**98.7**	**98.7**	**98.7**	97.7	**98.7**	97.0	**98.0**
14	*T. sinensis*	AB070483	97.7	**99.3**	**99.0**	97.3	**98.7**	**98.7**	97.7	**98.3**	**98.7**	**98.7**	**98.0**	**99.0**	**99.0**	**99.0**	97.3	**98.3**	96.7	97.7
15	*T. sinensis*	AB070482	97.7	**98.7**	**99.0**	97.3	**99.3**	**99.3**	97.7	**99.0**	**98.7**	**98.0**	**98.0**	**98.3**	**98.3**	**98.3**	97.3	**99.0**	96.7	**98.3**
16	*T. beneficus*	AB070502	96.3	96.0	96.3	95.6	96.0	96.0	96.0	95.6	96.0	95.3	96.3	95.6	95.6	95.6	95.6	95.6	95.3	96.0

firmed the presence of *T. sinensis* in Vrtojba and Ravnica. The parasitism rates were not calculated for Slovenia because 2015 was the first year of release.

Population genetic analysis

Cladistic analysis of 68 300bp long nucleotide sequences yielded 18 haplotype sequences based on 17 polymorphic loci. Overall haplotype diversity was Hd ± (SD) 0.855 (0.028), nucleotide diversity π ± (SD) 0.012 (0.001) and mean number of pairwise differences was MNPD 3.554.

Ninety-five percent of intraspecific Kimura-2-parameter distances between haplotypes were found within the interval of 0.3 – 2.7 % and the higher value was used as a cut-off to define the limit for species identification (25). Basic local alignment search (BLAST) with all determined haplotypes showed high similarity with available sequences of *T. sinensis*, and a few sequences of *Torymus beneficus* Yasumatsu & Kamijo were described and deposited in NCBI GenBank by Yara (2004) (31) (Table 2).

DISCUSSION

In total 10,590 females and 5,295 males were released in Croatia, Hungary and Slovenia in 2014 and 2015. Within the area of release there are huge and unbroken complexes of sweet chestnut forests where *T. sinensis* will find suitable host and disperse freely in all directions. During the early years of the release, the population of *T. sinensis* will disperse slowly and, over the years, the dispersion will be faster and exponential (6, 14, 17). Italian experience has shown that two release sites 8 km away from each other will see the merging of the populations of the parasitoid in 5 years, two sites 20 km apart in 7 years (17, 32). The minimum release per site in Croatia, Slovenia and Hungary was 100 females/50 males according to the experiences of Italian colleagues (15). Propagule pressure is a combined measure of the number of individuals of a species released (propagule size) and the number of release events (propagule number) (33, 34). As the number of releases or the number of individuals released increases, propagule pressure also increases. To increase the propagule pressure and the possibility of a successful establishment of *T. sinensis* populations, up to 1,200 females were released on some sites in Croatia (with additional release of 800 females on the same site) and 200 females were released again on the same site in Hungary in 2015. This has proven to be an efficient method as the population in Pazin, Croatia has been very quickly established. In the forest of an area of 13 ha after only two years parasitism rate has been 76 %, while similar parasitism rates have been observed on Italian sites after 6-7 years after releasing 100 females (17). Our experience with the release of a high number of females/males on one locality shows that it is possible to achieve high parasitism rates only one

year after the release (two releases). Previous research on parasitoids in Pazin has not shown the presence of *T. sinensis* (12), so present high parasitism rate can be attributed to the release of this parasitoid species. Pazin site will be used as a first collecting site for *T. sinensis* in Croatia and Tornyiszentmiklós will be used in Hungary. Molecular analysis has also given unexpected results: on two sites in Slovenia (Vrtojba and Ravnica) and two sites in Croatia (Tunel Učka, Samobor) *T. sinensis* was identified although there was no official and registered release on these sites. Morphological identification has also shown presence of *T. sinensis* on several sites across Slovenia. This presence of *T. sinensis* could be a result of unregistered release or natural spread from Italy where the introduced parazitoid has been widely released in the last 10 years (17). This finding will only facilitate and speed up the establishment and spread of *T. sinensis* populations in Croatia and Slovenia as this and the released populations will merge and disperse.

According to the high similarity of obtained haplotype sequences with the COI barcode sequences of *T. sinensis* deposited in GenBank, it can be concluded that the identification of analysed specimens was positive. However, similarity with a few sequences of *T. beneficus* was also observed (Table 2). A phylogenetic similarity between these two species was already reported in eastern Asia (31, 35), where a late-spring strain of *T. beneficus* was found to be phylogenetically closely related to *T. sinensis*. The same author reported a higher level of hybridization between these two taxa, indicating a relatively recent speciation and a low level of genetic divergence, the fact that can complicate species delimitation even by means of molecular biology.

Prior to the release of *T. sinensis*, all advantages and disadvantages of the introduction of this alien biocontrol species into natural stands in Croatia, Hungary and Slovenia have been considered. The introduction of *T. sinensis* as a viable management option for biological control of *D. kuriphilus* with the identification, assessment and weighing up of all adverse and beneficial effects of the introduction of a biological control agent (especially an alien species) was discussed prior to the introduction (5, 9, 36, 37). Croatia, Hungary and Slovenia have taken into account all known environmental risks, as well as the benefits, and decided to release *T. sinensis* into natural habitats although some references point out insufficient data of performing accurate risk assessment on *T. sinensis* (9, 37). *Torymus sinensis* is considered specific to *D. kuriphilus* (6), but recent research has found possible shift to non-target species particularly onto oak gall wasp, *Biorhiza pallida* (37). However, the evidence provided by the authors are too weak to make serious conclusions and further research is in need to confirm this host shift to oak gall wasps. These risks and shifts to non-target species are difficult to quantify and there is a possibility that they would be present in the *T. sinensis* range in the future.

However, all the experiences and results from Japan, the USA, Italy and France (6) have shown that *T. sinensis* is an effective biocontrol agent and that it manages to supress the populations of *D. kuriphilus* and the damage it makes to sweet chestnut forests and orchards. In Croatia, Slovenia and Hungary native *Torymus* species have shifted to *D. kuriphilus* (12, Matošević, Melika and Kos unpublished data) and our future research must focus on interactions between these native parasitoid species and the introduced *T. sinensis*. Nevertheless, we consider *T. sinensis* to be a safe and efficient biological control against *D. kuriphilus*, but further research on its spread, parasitism rates and possible shifts onto non-target species must be done.

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