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Genotypic characterization of *Torymus sinensis* (Hymenoptera: Torymidae) after its introduction in Tuscany (Italy) for the biological control of *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae)

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Abstract

Torymus sinensis Kamijo is an alien parasitoid that is used in many areas of the world for biological control the Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu. In Italy, this parasitoid was imported from Japan in 2003 and subsequently multiplied and released throughout the country. In this study, a phylogenetic investigation was carried out on insects from three different sites in northern Tuscany (Italy). Moreover, the possible hybridization between *T. sinensis* and some native *Torymus* species was evaluated. The conserved region 18S rRNA gene and the hypervariable ITS2 (*Internal Transcribed Spacer 2*) region of the ribosomal cistrone were selected as molecular markers. The DNA of individual adult insects was amplified with specific primers and the subsequent sequencing did not produce readable sequences. This led us to hypothesize that there might be more haplotypes inside a single insect. Consequently, the amplification products obtained previously were cloned. Sequencing the amplified products, after cloning, ruled out any hybridization between *T. sinensis* and the native *Torymus s*pecies, and also confirmed the presence of two haplotypes for the Tuscan population of *T. sinensis* both for the region of the 18S rRNA gene as well as for the ITS2 region.

Key words:

Molecular marker, haplotype, parasitoid, Internal Transcribed Spacer 2, hybridization

Introduction

Since ancient times chestnut tree cultivation has played an important role in European silviculture from an economic and environmental point of view (Aebi et al. 2007, Conedera et al. 2004a and 2004b), although this importance has changed over time and varies in different regions (Conedera et al. 2016). Many phytosanitary threats have affected the European chestnut cultivation. The most recent began in 2002 and was the Asian chestnut gall wasp (ACGW) *Dryocosmus kuriphilus* Yasumatsu, which was accidentally introduced from China into Europe. Already widespread in Korea, Japan and USA between the 1940s and 1970s (Gibbs et al. 2011), the first European record of ACGWs was in Piedmont (Italy) in 2002 (Brussino et al. 2002). From here, it spread out rapidly in Italy, and Turkey (Çetin et al. 2014, Avtzis et al. 2018).

The damage caused by ACGWs, whose parthenogenetic females lay their eggs in both leaf and reproductive buds, is due to the gall development with consequent severe production losses and a reduction in the photosynthetic surface of the leaves. Resistant chestnut ecotypes or the chemical control (EFSA 2010) have been used to control ACGWs, but only the introduction of an alien parasitoid from China, *Torymus sinensis* Kamijo (TORYSI) has led to stable results.

TORYSI is univoltine like its host, but a diapause of 12 months can occur in some larvae probably because of host deficiency (Ferracini et al. 2015). TORYSI has been introduced successfully into Japan and USA for the biological control of the ACGW (Gibbs et al. 2011). In 2005, TORYSI adults were first released in Piedmont (Quacchia et al. 2008) and then all over Italy (Quacchia et al. 2014). The first release of TORYSI in Tuscany was in 2010, as part of a regional ACGW biological control project (Conti et al. 2014).

The population of TORYSI introduced into Italy was imported from Japan, where in 1975 TORYSI was released to support the control activity exerted by a native torymid, *T. beneficus* Yasumatsu et Kamijo (Moriya et al. 2003). Later, using molecular markers (cytochrome oxidase subunit I [COI], ribosomal internal transcribed spacers 1 and 2 [ITS1 and ITS2]), Yara et al. (2007) demonstrated

that in one specimen hybridization had occurred between a female of *T. beneficus* and a male of TORYSI. This suggests that the hybridization is possible although quite rare.

DNA markers are used for assessing genetic diversity, identifying haplotypes and predicting migration and colonization (Salvato et al. 2002, Llewellyn et al. 2003, Margonari et al. 2004, Bosio et al. 2005, Behura 2006, Guo et al. 2017). Molecular markers are utilized to identify the phylogeny and biogeography of insect populations and to understand the means of evolution and evolutionary trajectories (Luque et al. 2002, Chatterjee and Mohandas 2003, Mohandas et al. 2004, Prasad et al. 2004). The main applications of molecular markers are: mating, parentage and kinship, insect plant interaction, insect pathogen interaction and insect ecology study. Molecular analysis allows a sample to be identified independently of the sex and the stage of the biological cycle. DNA-based techniques have thus proved particularly useful in the study of the taxonomic and phylogenetic relationships of insects (Caterino et al. 2000).

Many molecular markers have been used in various studies and several authors have reviewed the various marker techniques (Lehmann et al. 1997, Kuhner et al. 2000, Black et al. 2001, Nagaraju et al. 2001, Brumfield et al. 2003, Morin et al. 2004).

Ribosomal DNA (rDNA) is the most widely used nuclear sequence in evolutionary analyses. Thanks to its high rate of evolution, the ITS regions flanking the 18S, 5.8S and 28S regions, ITS1 and ITS2, have been used in phylogenetic inference for closely related taxa (Miller et al. 1996) and phylogeographical and other population genetic studies (Navajas et al.1998, Ji et al. 2003, Volkov et al. 2003, Long et al. 2004, Mahendran et al. 2006, Yara 2006, Kumar et al. 2018, Li et al. 2018).

The aim of this paper was to investigate the phylogeny of *T. sinensis* by comparing ITS2 sequences. We also investigated whether TORYSI populations in Tuscany (Italy) have undergone hybridization with native species belonging to the same genus.

Materials and Methods

Study sites

Three sites in northern Tuscany (Italy) were chosen for the samplings of ACGW galls (Fig. 1): Fosdinovo (FOS), Capezzano Monte (CAPE) and Catagnana-Barga (CATA). Two of these sites, Fosdinovo and Capezzano Monte, face the Mar Ligure coast while Catagnana-Barga is in a valley of the river Serchio. The Apuanian Alps separate Fosdinovo and Capezzano Monte from Catagnana-Barga.

Insect collection

In order to collect specimens of ACGW parasitoids, about 400 undisclosed galls were collected between 3 and 9 March 2016 in each of the three sites.

The galls were split into four plastic containers with a holed lid for each sampling site and maintained at room temperature and humidity. Twice a week, until mid May, the containers were monitored to observe parasitoid emergence. The specimens were captured, placed individually into a vial, labelled and frozen (-20°C). Given that TORYSI adults were the most common specimens, 50 of them were separated from the other parasitoids and labelled according to their origin with the following abbreviations: FOSX (Fosdinovo), CAPEX (Capezzano Monte), CATAX (Catagnana-Barga) where X is a literal and/or numeric code of the isolate. These individuals made up the stock for genetic analysis.

Dichotomous keys were used to identify the species of parasitoids (Askew, 1961; Gibson and Fusu, 2016; Graham, 1969; Vere and Gijswijt, 1998; Zerova, 1978) as well as for the comparison with type material available in the Department of Agriculture, Food and Environment (DAFE), University of Pisa and the Department of Agriculture, Food, Environment and Forestry (DAFEF), University of Florence.

DNA extraction

Genomic DNA was extracted from individual insects using the *Quick*-DNA Miniprep Plus Kit (Zymo Research, USA) following the manufacturer's instructions. The concentration of each DNA

sample was measured using a WPA biowave DNA spectrophotometer (Biochrom Ltd., Cambridge, England), and their integrity was evaluated by agarose gel electrophoresis. The DNA was stored at -20°C.

PCR primer design

The primer pairs for identifying the haplotypes of the partial region of 18S rDNA (To18SA and To18SB), were designed from nucleotide sequences (acc. Nos. MH543348 and MH5433489) using Primer3 software (Applied Biosystems) as reported in Table 1. The primers for *ITS2* were constructed in homologous regions, after CLUSTALW (Thompson et al., 1994) multi-alignment of sequences selected by BLASTN analysis of *Torymus sinensis* genes for *5.8S rRNA, ITS2, 28S rRNA*, partial and complete sequence, isolate*: CK15* (acc. no. AB200273); *Trichogramma minutum TmMS16 ITS1, 5.8S ribosomal RNA* gene, and *ITS2*, complete sequence (acc. no. AY374440); *Leptocybe invasa* voucher *Li_CN_1 5.8S ribosomal RNA* gene, partial sequence*; ITS2*, complete sequence*;* and *28S ribosomal RNA gene*, partial sequence (acc. no. KP143962); *Quadrastichus mendeli 5.8S ribosomal RNA* gene, partial sequence*; ITS2*, complete sequence and *28S ribosomal RNA* gene, partial sequence (acc. no. KF879806); and *Ooencyrtus pityocampae* haplotype *2f 5.8S ribosomal RNA* gene, partial sequence*; ITS2*, complete sequence and *28S ribosomal RNA* gene, partial sequence (acc. no. KM527088) (Table 1).

PCR amplification

Amplification was carried out by conventional PCR in 20 µl reactions containing 1x 10X DreamTaq Buffer (Thermo Fisher Scientific, USA) 0.5 µM of each primer (Table 1), 1U of DreamTaq (Thermo Fisher Scientific, USA), and 20 ng of template DNA. PCR was run in a PCR system 2700 (Applied Biosystems, USA): Thermocycling consisted of an initial denaturation step at 95°C (5 min), which was followed by cycles for:

ToITS2 (95°C for 30 sec, 50°C for 40 sec and 72°C for 40 s) 40 cycles;

To18SA (95°C for 30 sec, 54°C for 30 sec and 72°C for 30 s) 30 cycles;

To18SB (95°C for 30 sec, 56°C for 30 sec and 72°C for 30 s) 30 cycles;

final extension step at 72°C (10 min).

All reactions were checked for amplification by gel electrophoresis.

Amplified DNA sequences were directly inserted into a pGEM-T Easy Vector System (Promega, USA). Colony PCR was performed on putatively transformed colonies using M13Forward and M13Reverse as primers. The clones that showed inserts with different molecular weights using gel electrophoresis analyses were sequenced by automated sequencing (MWG Biotech, Ebersberg, Germany). The sequences were analysed using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to identify them in the GeneBank.

Phylogenetic analyses

All sequences were multi-aligned using the CLUSTALW program (https://www.genome.jp/tools-bin/clustalw). Phylogenetic trees were built using the MEGA7 program (Kumar et al. 2016). The evolutionary relationship was estimated based on the statistical model Neighbour-Joining (in MEGA7 program) with a bootstrap number equal to 1000.

Results

Gall parasitoids

A total of 1048 parasitoids emerged from the galls collected in the three sites (Table 2). TORYSI was the most abundant species in the three sampling sites. The other species were polyphagous native parasitoids.

DNA amplification with the *universal primer 18S rRNA gene*

DNA of *T. sinensis* isolates 3 and 14, collected in Fosdinovo (FOS), were amplified with *universal primer 18S rRNA* gene and two different bands were highlighted, as shown in Fig. 2.

The PCR products were cloned and sequenced. The aligned sequences revealed two haplotypes, A and B, for the partial *18S rDNA* gene, as shown in Fig. 3.

Phylogenetic analysis of the sequences confirmed the occurrence of two clusters, the ToSA and ToSB haplotypes (Fig. 4).

DNA amplification with the primer for *Internal Transcribed Spacer* 2 (ITS2) and sequences analysis

DNA amplification products with primer ToITS2 (Table 2), extracted from *T. sinensis* collected at our three sites, and *T. flavipes* and *T. auratus* from Fosdinovo, were cloned and, after PCR colony screening, clones showing different molecular weights (Fig. 5) were sequenced.

After sequence alignment, a phylogenetic tree (Fig. 6) was constructed using all the sequences obtained in this study along with the sequences of *T. sinensis, T. auratus* and *T. flavipes* (from specimens collected in the study site), *T. geranii* (species with a Palearctic distribution collected from ACGW galls in several regions of Italy) and *T. beneficus* (Japanese species). The sequences of *T. geranii* and *T. beneficus* were retrieved from public databases. We used the ITS2 of *Bombyx mori* as the outgroup.

All sequences isolated in this study belonged to one of two clusters, C and D. All individuals felt into one of the two clusters, except for the capeA isolate which showed ITS2 sequences from both clusters.

Discussion

The role of *T. sinensis* as the main parasitoid in ACSW galls was confirmed in the three study sites. However, despite the restricted time frame of the sampling, other native parasitoids also emerged. Many native parasitoids, especially all chalcidoidea hymenopterans, have adapted to the ACGW larvae in Italy as well as in other sites in Europe where the ACSW was introduced (Quacchia et al. 2008, Matošević and Melika 2013). In fact, they have shifted to the new host and have aided *T.*

156 *sinensis* in its role as a biological control. However, the parasitism rate of these native parasitoids,
157 which are frequently associated with oak gall wasps, is generally low. The native parasitoids
158 observed in the sampling include species already observed in other sites in Tuscany (Panzavolta et
159 al. 2013, Panzavolta et al. 2018).
160 For the phylogenetic investigations of *T. sinensis*, we used nuclear ribosomal DNA (rDNA) given
161 that it is present in many copies in every species and is known to provide insights into the
162 evolutionary history of different organisms (Nyaku et al. 2013, Costa et al. 2016, Zhang et al.
163 2017).
164 While the rRNA genes are conserved among the species, the intergenic spacers (ITS1 and 2) evolve
165 rapidly and have been widely used for intraspecific analyses of diversity in numerous organisms,
166 including animals and plants. The conserved region rDNA 18S has been extensively used for
167 evaluating relationships among taxa (Gomulski et al. 2005, Fritz 2006, Nyaku et al. 2013,
168 Venkatesan et al. 2016).
169 The genetic analysis carried out on some of the TORYSI adults that had emerged from the galls,
170 showed the presence of two haplotypes. No nucleotide difference within each cluster was found
171 with the specific haplotype primers (To18SA and To18SB), differently from Nyaku et al. (2013)
172 who found two variants of the 18S rDNA when they were working on *Rotylenchulus reniformis*, a
173 plant parasitic nematode.
174 The phylogenetic analysis using ITS2 sequences, showed that the specimens of *T. sinensis* that we
175 isolated can be differentiated from the two native species of the same genus (*T. auratus* and *T.*
176 *flavipes*) collected in our sampling sites. This analysis also confirms that *T. sinensis* and *T.*
177 *beneficus* belong to the same cluster, confirming the results of Montagna et al. (2018). This analysis
178 also showed that all our isolates belonged to one of the two clusters, except for the capeA isolate
179 which showed ITS2 sequences of both clusters (Supp. Fig. S1)
180 These results suggest that, in the area of this study, *T. sinensis* imported to Italy did not hybridize
181 with the native *Torymus* species, such as *T. auratus* and *T. flavipes*. In fact, none of our isolates

share the ITS2 sequence with these species. However, this is also true for *T. geranii*, whose DNA sequence is in a data bank derived from specimens collected in two regions in the north of Italy (Piedmont and Liguria). The absence until now of hybridization with native species is a positive feature in the evaluation of the environmental impact of TORYSI. However, in order to minimize the environmental risks routine analyses for intentionally-introduced natural enemies should be carried out on a larger scale and implemented with other evaluations on behavioural aspects. For instance, the host range of TORYSI was recently demonstrated to be broader than that reported in the literature (Ferracini et al. 2015), since it is attracted by non-target hosts other than *D. kuriphilus.*

Supplementary Data

Supplementary data are available at Annals of the Entomological Society of America online.

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Conflicts of Interest

The authors declare no conflict of interest.

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Table legends

Table 1. List of *Torymus sinensis* specific primer sequences used in PCR assays.

Table 2. Species and number of parasitoids emerged from the ACGW galls in 2016

Figure legends

Fig. 1. Google map of the study sites: FOS=Fosdinovo, CAPE=Capezzano Monte, CATA= Catagnana-Barga.

Fig. 2. Electrophoretic analysis of PCR products of *T. sinensis* isolates 3 and 14 (fos3, fos14) with the *universal primer 18S rRNA* gene

Fig. 3. Alignment of 18S rRNA gene sequences: Haplotype A (ToSA) and Haplotype B (ToSB) of *T. sinensis* collected in Fosdinovo (FOS).

Fig. 4. Molecular phylogenetic relationship between haplotypes of sequences of *T. sinensis*: FOS, collected in Fosdinovo, CAPE, collected in Capezzano Monte and CATA, collected in Catagnana-Barga. In the molecular phylogenetic relationship, X, Y and Z represent respectively: the

provenance of sample (X), its name (Y) and the clone (Z) (for example capesnow1 is provenance "cape", the sample "snow" and the clone "1"). The molecular phylogenetic relationship between haplotypes was estimated on the basis of a *Neighbour-Joining* statistical model. The numbers by the nodes represent the *bootstrap* support percentage, estimated with 1000 replicates in MEGA7. In the tree, the branches of two haplotypes (A and B) are highlighted respectively in red and green. The sequence of *Bombyx mori* was used as an outgroup.

Fig. 5. Electrophoresis of PCR colony screening of 12 colonies

Fig. 6. Molecular phylogenetic relationship among sequences of *T. sinensis*: FOS, collected in Fosdinovo, CAPE, collected in Capezzano Monte and CATA, collected in Catagnana-Barga, TA: *T. auratus*, TB: *T. beneficus;* TF: *T. flavipes*, TG: *T. geranii*, TS: *T. sinensis* sequence from the gene bank. TA14_2gb|LT821706|, TA14_1gb|LT821705|, TG15Q_1gb|LT821715|, TG12C_1gb|LT821714|, TSgb|LT821666| in the gene bank were from Italy, the remaining specimens came from Japan.
In the aligment, X, Y and Z represent respectively: the provenance of sample (X), its name (Y) and the clone (Z) (for example cape1A5 is provenance "cape", the sample "1A" and the clone "5"). In the tree, the sequences from the database are highlighted in red, and the branches of two haplotypes C and D are highlighted respectively in blue and pink. The evolutionary relationship was estimated by the statistical neighbor-joining model and the bootstrap was estimated with 1000 replications with the MEGA7 program. The sequence of *Bombyx mori* was used as an outgroup. Asterisks represent a bootstrap of more than 50%.