Evidence for an elevated aspartate pKa in the active site of human aromatase

Title

Molecular species delimitation of the Asian chestnut gall wasp biocontrol agent released in Italy

Short title

Molecular delimitation of torymid species

Author Name

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Abstract

Molecular species recognition and identification, based on the mitochondrial *cox1* and on the nuclear ITS2, were performed on individuals of *Torymus sinensis* collected in Italy, on its close relative *T. beneficus* and on native torymids. The automatic-gap-discovery (ABGD) analyses correctly separate almost all morphospecies. On the basis of *cox1*, individuals of late-spring *T. beneficus* clustered with *T. sinensis,* and those identified as early-spring *T. beneficus* were recognized as a separate entity. Whereas, *T. beneficus* ecotypes clustered with *T. sinensis* on the basis of ITS2. Coalescent tree-based methods confirmed these results. The *cox1*-based recognition of early-spring *T. beneficus* as a separate phylospecies led us to conclude that this taxon deserves to be treated as a valid species, whereas individuals identified as late-spring *T. beneficus* might be considered as part of *T. sinensis*. Morphological identification and BLAST analyses confirmed that no *T. beneficus* was imported into Italy to control *Dryocosmus kuriphilus.*

Keywords

Classical biological control, *Dryocosmus kuriphilus*, molecular taxonomy, *Torymus beneficus*, *Torymus sinensis*, *Wolbachia*

Introduction

The Asian chestnut gall wasp (ACGW), *Dryocosmus kuriphilus* Yasumatsu, 1951 (Hymenoptera: Cynipidae), native of China, was considered to be a major pest of chestnut (*Castanea* spp.) by the mid-20th century. It established itself in several countries, being reported in Japan (1941), Korea (1958), the United States (1974), Nepal (1999), and Canada (2012). In Europe, it was first recorded in Italy in 2002, and is now reported in 19 European countries (Delalić 2016; EPPO 2016; Michaelakis 2016; Radócz 2016).

This gall wasp induces the formation of greenish-red galls, which develop at the time of budburst in the following early-spring, suppressing shoot elongation and causing a gradual decline in the vigor of the host plants (EFSA 2010). Severe nut production losses due to the development of galls were estimated to reach up to 85% in northern Italy (Bosio et al. 2013; Battisti et al. 2014).

In Japan, the indigenous parasitoid *Torymus beneficus* Yasumatsu & Kamijo, 1979 (Hymenoptera: Torymidae) was used as a biological control agent of the ACGW; however, it was not able to suppress chestnut gall wasp populations, thus requiring the introduction of the congeneric species *T. sinensis* Kamijo, 1982 from China (Yara 2006). These two species, *T. sinensis* and *T. beneficus,* are difficult to separate on the basis of either their morphology or ecological traits. The ratio of the ovipositor sheath length to the lateral length of the thorax (O/T ratio), and the emergence period were used to distinguish empirically the two species (Ôtake, 1987; Moriya et al. 1992). However, the O/T ratio was shown not to be completely reliable (Yara 2004) and applicable for female adults only, and the discrimination based on the emergence period may be hampered by slight differences observed between the two species and by the fact that two ecotypes of *T. beneficus* have been described, namely early-spring and late-spring types, each showing different emergence periods (Yara et al. 2000). Molecular markers were developed to distinguish the two species, such as isozyme and DNA markers, the latter targeting nuclear and mitochondrial DNA (Yara 2004; Yara 2006; Yara & Kunimi 2009). Additional problems with species discrimination are based on hybridization that was observed between *T. sinensis* and both ecotypes of *T. beneficus* (Toda et al. 2000; Yara et al. 2000; Yara et al. 2010). These hybrids are known to have intermediate morphological features in respect to the parental species. Hybridization between males of *T. sinensis* and females of *T. beneficus* was hypothesized on the basis of mtDNA showing a closer relationship between late-spring *T. beneficus* and *T. sinensis* than to early-spring *T. beneficus* (Yara 2004). Hence, definitive differentiation between *Torymus* species and hybrids has not yet been achieved (Yara & Kunimi 2009; Yara 2014). Different possible hypotheses could be formulated to explain the phylogenetic pattern reported in Figure 2 in Yara (2004), mainly: *i*) inadequate taxonomy, *viz T. beneficus* late spring ecotype is synonym of *T.*

sinensis, as suggested by the great variability on the O/T ratio of these species (Yara 2004); *ii*) incomplete lineage sorting; and *iii*) the occurrence of endosymbiont-mediated mtDNA introgression causing discordance between species and gene trees in the *T. sinensis – T. beneficus* group, as in the case of the parasitoid wasps of the genus *Diplazon* and the alpha-proteobacterium *Wolbachia* (Klopfstein et al. 2016).

After 15 years from the first report of the ACGW in Europe, and despite the species richness of the recruited parasitoid community, parasitism by native natural enemies is still low (Matošević & Melika 2013; Quacchia et al. 2013; Alma et al. 2014; Francati et al. 2015; Colombari & Battisti 2016). For this reason, a classical biological control approach was undertaken in Europe similar to those in Japan and in the USA (Moriya et al. 2003; Cooper & Rieske 2011). *Torymus sinensis* specimens imported from Japan were released in Italy starting in 2005 (Quacchia et al. 2008; Ferracini et al. 2015a), and afterwards in other European countries (Paparella et al. 2016). It proved to settle successfully in the chestnut-growing areas containing the ACGW outbreaks especially in northern Italy where it was initially released. Moreover, after a ten-year period, a differentiation in observed behaviors was recorded (e.g., prolonged diapause and host range expansion; Ferracini et al. 2015a, Ferracini et al. 2017).

Even if no evidence of divergent emergence periods for *T. sinensis* was ever recorded in Italy, but due to the difficulty in discriminating *T. beneficus*, *T. sinensis*, and hybrids, investigations on the biocontrol agent of the ACGW released in Italy were carried out. In particular, using molecular approaches and species delimitation tools, three major goals are addressed in the present study: *i*) test the congruence between morphospecies and phylospecies in the introduced and indigenous *Torymus* species; *ii*) establish whether *T. beneficus* individuals or hybrids were initially released in Italy together with Japan-imported *T. sinensis* specimens; *iii*) investigate the presence of *Wolbachia* in the examined species to provide preliminary information regarding the distribution of this bacterium within *T. sinensis – T. beneficus* late spring ecotype.

Materials and Methods

Ethics statement

All experiments were conducted in accordance with the legislation and guidelines of the European Union for the protection of animals used for scientific purposes (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm).

All experimental protocols using animals were approved by the *ad-hoc* Committee of DISAFA of the University of Torino. In addition, all necessary permits were in hand when the research was conducted.

Insects sampling, manipulation and morphological identification

From 2006 to 2016, putative *T. sinensis* adults emerging from withered chestnut galls collected in Italy and Japan were used for this study, along with native species of *Torymus* emerging from withered oak and chestnut galls collected in Italy, as described in Table 1 and Figure 1. (Fig. 1 here) More precisely, regarding *T. sinensis*, the DNA was extracted from: *i*) specimens emerged in Italy from chestnut withered galls collected in Japan and provided by the National Agricultural Research Center in Tsukuba (Quacchia et al. 2008); *ii*) the progeny of the adults used in the first releases in Italy which occurred during 2006 and 2008; *iii*) individuals emerging from withered chestnut galls collected in Piedmont (Italy) between 2014 and 2016, where the parasitoid was released for the first time, together with specimens emerging after a two-year diapause; and iv) three individuals, collected from oak galls of the gall wasp *Biorhiza pallida* (Olivier, 1791). Native torymids, emerging from galls of *D. kuriphilus*, *B. pallida*, *Cynips divisa* Hartig, 1840 and *Cynips quercusfolii* Linnaeus, 1758 collected in Aosta Valley, Liguria, and Piedmont regions of north-western Italy, were also included in the present study. Collections were performed both by hand and with the aid of lopping shears. Galls were isolated in plastic containers and stored in outdoor conditions until parasitoid emergence, according to the method described by Ferracini et al. (2015b). Among the emerged parasitoids, torymid species were identified by using specific dichotomous keys reported in De Vere Graham & Gijswijt (1998) and Kamijo (1982), and by comparison with voucher specimens deposited at the DISAFA-Entomology laboratory, Italy. We were able to include *T. affinis* (Fonscolombe, 1832), *T. auratus* (Müller, 1764), *T. cyaneus* Walker, 1847, *T. flavip*es (Walker, 1833), and *T. geranii* (Walker, 1833) (Table 1).

DNA extraction, PCR and sequencing

Chelex DNA extraction according to Kaartinen et al. (2010) was performed on metathoracic legs, dissected from the specimens. All the extracted specimens were stored in ethanol and preserved in the DISAFA collections with vouchers reported in Table 1. Samples were subject to amplification of the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene, and the nuclear region of the internal transcribed spacer 2 (ITS2) between *5.8S* rRNA and *LSU* rRNA genes. For *cox1* amplification, primers COI pF2 (Kaartinen et al. 2010) and HCO (Folmer et al. 1994) were used with the following conditions: an initial denaturation step of 2 min at 94°C, followed by 5 cycles of 30 sec at 94°C, 1 min at 45°C, and 1 min at 72°C, then 35 cycles of 30 sec at 94°C, 1 min at 50°C, and 1 min at 72°C; a final extension step of 7 min at 72°C was applied. PCR for ITS2 was carried out with primers ITS2f/r as described by Campbell *et al.* (1993). The obtained PCR products were purified with the commercially available kit (GenElute™ PCR Clean-Up Kit, Sigma-Aldrich) and sequenced (Genechron, Rome, Italy).

Electropherograms were manually checked using Geneious Pro 8.1 (Biomatters Ltd., Auckland, New Zealand); no heterozygotes were recovered in ITS2 electropherograms using a double peaks similarity of 95%. The nucleotide sequences were deposited in the European Nucleotide Archive with accession numbers LT821524 - LT821619 and LT821620 - LT821715, respectively for *cox1* and ITS2. To confirm the morphological identification of the specimens, *cox1* gene sequences were subjected to Nucleotide BLAST analysis against nr database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Orthologous sequences from *Torymus* spp., with the addition of *Bootanomyia* (=*Megastigmus*) *dorsalis* (Fabricius, 1798) as outgroup, were retrieved from GenBank (Table 1). The *cox1* dataset was aligned at the amino acid level using MUSCLE (Edgar 2004) and back translated at the nucleotide level, whereas the ITS2 dataset was aligned using MAFFT (Katoh et al. 2005) with E-INS-i strategy. Poorly aligned positions in ITS2 alignment were trimmed with Gblocks (Castresana 2000) using the strategy adopted by Sassera et al. (2010) for a less stringent selection.

Species delimitation analyses

Different methods, with no *a priori* information on the morphospecies, were adopted as described in previous studies (Montagna et al. 2016a, b). Briefly, the automatic barcode gap discovery tool (ABGD; Puillandre et al. 2012) and coalescent tree-based methods represented by the generalized mixed Yule-coalescent model (GMYC; Pons et al. 2006; Fujisawa and Barraclough, 2013) and its Bayesian implementation (bGMYC; Reid & Carstens 2012) were used. These methods were extensively used to recognize and delimit species (e.g., Cranston & Krosh 2015; Lecocq et al. 2015) as well as support the description of new insect taxa (e.g., Montagna et al. 2013; Montagna et al. 2016b).

ABGD analyses were performed on *cox1* and ITS2 aligned sequence datasets through the web interface (http://wwwabi.snv.jussieu.fr/public/abgd) with the uncorrected *p* distance (Collins et al., 2012; Srivathsan et al., 2012) and default settings for the remaining parameters.

The ultrametric tree required as input by the coalescent tree-based methods of species delimitation was inferred with BEAST (Drummond et al. 2012). Each alignment was analyzed with JModelTest 2 (Darriba et al. 2012) and the most suitable model of nucleotide evolution was selected using the Bayesian Information Criterion (Sullivan & Joyce, 2005). Identical haplotypes of conspecific specimens were removed from the datasets in order to outflank zero-length internal nodes in the trees. Phylogenetic reconstruction was achieved under the Bayesian framework using BEAST (Drummond et al. 2012), three independent runs were performed for each dataset with the following settings: length of the Markov chain of $10^{\wedge 8}$ generations; trees and parameters sampled every 1,000 generations; models of nucleotide evolution as obtained by the

model selection, *viz* GTR (Lanave et al. 1984) with gamma distribution (Γ) and proportion of invariable sites (I) in the case of *cox1*, K80 (Kimura, 1980) + I in the case of ITS2; uncorrelated relaxed clock type (Drummond et al. 2006); tree prior set on the Birth-Death Incomplete Sampling (Stadler 2009); other prior parameters were set to default values. The convergence of the runs was visually examined by TRACER (Drummond et al. 2012), and the burn-in fraction estimated accordingly. After the removal of the burn-in trees fraction, trees were resampled at lower frequencies in order to obtain 5,000 trees for each run, and then resampled trees were merged and the maximum clade credibility tree was obtained using TreeAnnotator (Drummond et al. 2012).

Single-threshold GMYC analyses and bGMYC analyses were performed using the R packages SPLITS and bGMYC respectively. The maximum clade credibility ultrametric tree previously inferred was used as input for GMYC; whereas, in the case of bGMYC, 30 randomly sampled trees among the 15,000 merged BEAST trees were used as input. The parameters of bGMYC analysis were: Markov Chain Monte Carlo length of 100,000 generations, sampled every 100 generations and a burn-in of 800 trees, leading to the estimation of the statistics on a sample of 6,000 trees.

Pairwise nucleotide *p*-distance within and between taxa were calculated using MEGA 6 (Tamura et al. 2013), missing data and gaps were excluded in the pairwise distance estimation and the standard deviation was estimated by 500 bootstrap pseudo replicates.

Wolbachia detection

In order to provide preliminary information regarding the distribution of *Wolbachia* within the *T. sinensis – T. beneficus* group, DNA isolates from all *T. sinensis* specimens were tested for the presence of this bacterium by PCR targeting the 16S rRNA gene by using the W-Spec f/r primer pair as previously described (Werren & Windsor, 2000), after whole body DNA extraction according to Raddadi et al. (2011). None of the PCRs targeting *Wolbachia* genes led to positive amplifications, with the exception of the positive control, and thus this result will not be reported in the *Results and Discussion* section but only commented in the *Conclusion*.

Results

Datasets description and molecular species identification

DNA was extracted from a total of 96 adult specimens (47 females and 49 males) collected from different localities and attributed to the following six species: *T. affini*s (n = 6)*, T. auratu*s (n = 2)*, T. cyaneu*s (n = 4)*, T. flavipe*s (n = 3), *T. geranii* (n = 2), and *T. sinensi*s (n = 79) (Table 1). A fragment of 427 bp of the mitochondrial *cox1* gene and ITS2 sequences,

ranging from 478 bp of *T. sinensis* to 638 bp of *T. affinis*, were obtained for 95 individuals. The morphological identification of the specimens collected in Italy was confirmed by a BLAST search on *cox1* gene sequences (99%-100% of identity with conspecific specimens). According to previously published ITS2 profiles (Yara 2006), all the *T. sinensis* collected in Italy possessed a homoallelic genotype, 57 individuals the 0/0, 14 individuals the -2/-2 and the specimen TsPBp13_1 collected in Piedmont from a gall of *B. pallida* the genotype -4/-4 (Table 2).

With the addition of orthologous gene sequences available in public repositories for the species *T. affinis*, *T. auratus*, *T. beneficus* (early- and late-spring ecotypes), *T. flavipes*, *T. geranii* and *T. sinensis*, the *cox1* and ITS2 datasets were composed of, respectively, 136 and 103 nucleotide sequences, which, resulted in 101 and 60 sequences for *cox1* and ITS2, respectively, after the removal of identical nucleotide sequences (as described in section *Species delimitation analyses* in Materials and Methods).

Distance-based species delimitation

ABGD analysis performed on the aligned *cox1* gene sequences retrieved a perfect match between the initial and the recursive partitions at nucleotide divergence ranging from 0.3% to 1.3%, and nine groups were identified (or putative molecular species), highly congruent with morphological identification, (Fig. S1). Particularly, *T. affinis*, *T. auratus*, *T. cyaneus*, *T. flavipes, T. geranii*, *Torymus* sp. and *B. dorsalis* (outgroup) were recovered as separate entities; specimens identified in the literature as late-spring *T. beneficus* grouped with *T. sinensis*, while, all the individuals identified as early-spring *T. beneficus* belonged to a separate group. All the specimens collected in Italy (details are reported in section *Insects sampling, manipulation and morphological identification* in Materials and Methods) grouped with specimens identified as late-spring *T. beneficus* and as *T. sinensis* retrieved by previous studies. Increasing the prior intraspecific nucleotide divergence to values between 2% and 3.6% (Figure S1), the initial and recursive partitions match in a second optimum, where the specimens identified as early-spring *T. beneficu*s were recognized in the same evolutionary unit with individuals of the late-spring ecotype and the closely related *T. sinensis*. The close relationship of *T. sinensis* and *T. beneficus* (both ecotypes) is also confirmed by the low between-taxa nucleotide *p*-distance of 4.8% (SD = 0.7%; Table 3). When the two ecotypes of *T. beneficus* are considered separately, the nucleotide *p*-distances between them and *T. sinensis* were 1.8% (SD = 0.4%) and 5.7% (SD = 0.9%), respectively in the case of late- and early-spring ecotypes (Table 3).

Similar results in the delimitation of the species were achieved also by ABGD analyses performed on the ITS2 dataset. Seven partitions were obtained from values of nucleotide *p*-distance ranging from 0.8% to 10%, with a match between the initial and recursive partitions (Fig. S2). In the case of ITS2, all the specimens identified as *T. sinensis* and the two

specimens of *T. beneficus* (early- and late-spring) clustered within the same group (the achieved result is not attributable to the adopted trimming strategy since no nucleotides of these two taxa were removed from the alignment); a similar behavior was observed also for *T. auratus* and *T. geranii*. Interestingly, the specimen of *T. geranii* from Tsukuba (Japan, AB200280) was assigned to a separate group in respect to the Italian conspecific specimens. The latter result is in agreement with the extremely high value of within-taxa nucleotide *p*-distance recovered for *T. geranii* (average 21%, SD = 1.7%; Table 3), in the range of between taxa values. Except for *T. geranii*, the intraspecific nucleotide *p*-distance calculated on ITS2 spans from 2.4% (SD = 0.5%) to 0%, respectively in the case of *T. affinis* and *T. beneficus* (one individual for each of the two ecotypes; Table 3); whereas the interspecific nucleotide *p*-distance range from 30.6% (SD = 2.1%) between *T. geranii* – *T. cyaneus* to 0.5% (SD = 0.2%) between *T. sinensis* and *T. beneficus* (Table 3).

Tree-based species delimitation

The topologies of the Bayesian consensus *cox1* and ITS2 trees, used as input for the tree-based species delimitation methods, were not totally congruent, as expected for closely related taxa (Figures 2 and 3). (Figures 2, 3 here)

On the basis of *cox1*, almost all the analyzed species were well supported and monophyletic, with a Bayesian posterior probability (BPP) of 1; the only exception is represented by the sequences identified as late-spring *T. beneficus* that clustered with *T. sinensis.* Two well supported clades (BPP of 0.97 and of 0.96), sub A and sub B in Figure 2, can be identified within the clade late-spring *T. beneficus* – *T. sinensis* (BPP = 1); however, a clear clustering pattern is not identifiable. All the specimens collected in Italy and identified as *T. sinensis* clustered within the clade *T. sinensis* – late-spring *T. beneficus*, no specimens of early-spring *T. beneficus* were imported into Italy to control the ACGW through the withered chestnut galls. Even the specimen TsPBp13_1, with genotype -4+G corresponding to that of early-spring *T. beneficus*, clustered within the clade *T. sinensis* – late-spring *T. beneficus* on the basis of *cox1* sequence, as to be expected considering the results of BLAST analysis. Conversely, relationships among the species groups, especially those between the complex *T. sinensis* – *T. beneficus* and the remaining species are not resolved on the basis of *cox1* gene. Moreover, in the case of the ITS2 tree, the Bayesian posterior probability associated with morphospecies was high but *T. beneficus* and *T. geranii* resulted paraphyletic.

Species delimitation analyses performed by implementing coalescent tree-based approaches led to almost identical results for both *cox1* and ITS2 markers. Regarding the *cox1* dataset, the GMYC model exhibited a significantly better likelihood than the null model ($\log L_{GMYC} = 822.6$, $\log L_{NULL} = 805.8$, $2\Delta L = 33.5$, χ^2 test *p-value* < 0.0001). At the threshold between Yule and Coalescent models, *viz* cladogenesis – anagenesis, seven maximum likelihood clusters (95% CI [7, 8]) and a total

of ten entities or putative molecular species (95% CI [10, 11]) were identified (Figure 2). Identical results were obtained by bGMYC, which identified 10 evolutionary units supported by a BPP ≥ 0.65 (Figure 2). These results are in accordance with those obtained by the distance-based approach. The morphospecies included in the present study, with the only exception of *T. sinensis – T. beneficus*, were identified by GMYC and bGMYC as belonging to separate entities. *Torymus beneficus*, as previously reported (Yara, 2004), resulted paraphyletic; the lineage with individuals assigned to early-spring phenotype resulted monophyletic and well isolated (BPP_{bGMYC} = 0.75), the sister of the clade *T. sinensis* – late-spring *T. beneficus* (BPP_{bGMYC} = 0.65). For the highest value of the confidence interval of entities recovered by GMYC (i.e., 11), obtained moving the Yule-Coalescent threshold towards the tips, the clade *T. sinensis* – late-spring *T. beneficus* is split into two distinct clades (Figure 2) with a clustering pattern of individuals not explicable either in terms of their geographic origin nor in terms of ecology. All the specimens identified as late-spring *T. beneficus* are grouped in the clade sub A (BPP = 0.97; Figure 2), possibly corresponding to subgroup 3 in Figure 2 of Yara (2004). However, the fact that individuals of *T. sinensis* (AB07483, AB07484) belonging to subgroups 1 or 2 in Figure 2 of Yara (2004) clustered here within the clade sub A with late-spring *T. beneficus* does not support this possibility, but more likely, that *T. sinensis* – late-spring *T. beneficus* represents a single species.

On the basis of ITS2 seven maximum likelihood entities have been identified (Figure 3) ($logL_{GMYC}$ = 434.1, $logL_{NULL}$ = 431.1, $2\Delta L$ = 6; *p-value* = 0.049), but a wider 95% confidence interval in respect to that achieved on the basis of the *cox1* marker is recovered (95% CI [3-34]). bGMYC reached almost identical results of those of GMYC, identifying five entities with BPP ≥ 0.65 and seven with BPP > 0.5 (Figure 3). It is noteworthy that both ecotypes of *T. beneficus* (represented by one specimen each) fall into a single group with *T. sinensis* (BPP = 0.67), confirming the results achieved by ABGD. Two separate and well-supported entities could be recognized within the clade composed of *T. sinensis – T. beneficus*. Apart from the *T. beneficus – T. sinensis* clade, *T. geranii* resulted paraphyletic, while *T. affinis*, *T. flavipes* and *T. cyaneus* resulted monophyletic and recognized as separate entities.

Discussion

Taking advantage of the recent introduction of *T. sinensis* in Italy as a biocontrol agent of the ACGW, the present study is mainly focused on testing the congruence between morphospecies and putative molecular species (or phylospecies) of the highly morphologically similar *T. sinensis – T. beneficus* complex as well as native torymids. The results achieved by the previous studies attempting to distinguish *T. sinensis* from *T. beneficus* using molecular markers (Yara 2004, Yara 2006,

Yara & Kunimi 2009) prompted us to adopt rigorous and recently developed species delimitation tools (i.e., ABGD, GMYC and bGMYC) in order to test the species status of these two taxa and that of the native torymids. Moreover, since the clustering pattern of *T. sinensis* and *T. beneficus* previously obtained by *cox1* gene sequences was congruent with endosymbiont-mediated mtDNA introgression, specimens of *T. sinensis* collected in Italy were tested for the presence of *Wolbachia*, widespread in invertebrates and recognized as the master manipulator of their reproduction (Werren et al. 2008). None of the analyzed individuals showed the presence of this bacterium. However, since topologies obtained using *cox1* and ITS2 are discordant in respect to *T. sinensis – T. beneficus* group, *viz T. sinensis* and *T. beneficus* (early- and late-spring) belong to the same clade using ITS2 but early-spring *T. beneficus* belong to a different clade using *cox1*, the presence of *Wolbachia* during the evolution of the group followed by a secondary loss cannot be excluded on the basis of the present study. Moreover, the possibility that others vertically inherited endosymbionts, as "*Candidatus* Cardinium" (Zchori-Fein and Perlman, 2004), influenced the evolution of the group was not investigated in this study.

Using *cox1* and ITS2 markers, all the adopted species delimitation approaches, represented by nucleotide distance and coalescent tree-based methods, agree in recognizing as separate phylospecies all the native morphospecies (Figures 2 and 3). Interestingly, the ITS2 sequence of *T. geranii* available in GenBank (AB200280) did not cluster with conspecific specimens collected in Italy, and due to its high value of nucleotide *p*-distance relative to the other conspecific (>20%), it could be considered as belonging to a different taxon (Figure 3). Regarding the *T. sinensis – T. beneficus* (early- and late-spring ecotypes) complex, on the basis of *cox1* marker all specimens of early-spring *T. beneficus* were identified as a separate phylospecies; this result let us hypothesize that the taxon deserves to be treated as valid species. Whereas, since individuals identified as late-spring *T. beneficus* resulted in a well-delimited group together with all *T. sinensis*, they might be considered as part of this latter species (Figure 2). This result is further confirmed by the low value of the *cox1* mean nucleotide *p-distance* between *T. sinensis* and the late-spring *T. beneficus* (1.8%, SD = 0.4), which is in the range of the intraspecific nucleotide distance values estimated in this study for the other analyzed *Torymus* species (Table 3).

Using ITS2 marker, *T. beneficus* (represented only by two individuals for which the corresponding *cox1* sequences are not available in public repositories) and *T. sinensis* (39 individuals) belong to the same evolutionary unit (Figure 3). The two well-supported entities identified by bGMYC within the *T. sinensis – T. beneficus* group do not match any apparent geographic or ecological pattern. The results achieved using this marker possibly suggests that ITS2 profile is not reliable to distinguish species and ecotypes within the *T. sinensis* species complex; however, since to our knowledge, *cox1* and ITS2

sequences of the same *T. beneficus* individual are not present in public repositories, a conclusion cannot be reached using the available data.

The values of nucleotide *p*-distance, the achieved tree topologies, and the species delimitation results are congruent with the following scenarios: *i*) a recent origin of the taxa of *T. sinensis – T. beneficus* complex, in which a complete lineage sorting has not yet been achieved; *ii*) the presence of two delimited species, *viz.* early spring *T. beneficus* and late spring *T. beneficus – T. sinensis,* with the resulting incorrect identification of individuals assigned to *T. beneficus* late spring; or, *iii*) a single species requiring synonymy.

In conclusion, the achieved results are far from being conclusive and an integrative taxonomy analysis with an increased number of specimens and based on a multi-gene approach is required to definitively solve the boundary between these two sister species. Besides, our study confirms that no individuals of the early-spring *T. beneficus* ecotype were imported from Japan to control the ACGW, *Dryocosmus kuriphilus*.

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Disclosure

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figure Legends

Figure 1. Collection sites of the specimens analyzed in the present study.

Figure 2. Species delimitation analysis based on *cox1* gene sequences. A Bayesian ultrametric tree inferred from the *cox1* gene sequence dataset, after the removal of identical haplotypes. *T. beneficus – T. sinensis* specimens are highlighted with different colors as follow: green = *T. sinensis*, from the present study; yellow = *T. sinensis,* from GenBank (Table 1); light blue = late-spring *T. beneficus* ecotype, from GenBank (Table 1); fuchsia = early-spring *T. beneficus* ecotype, from GenBank (Table 1).

Clades corresponding to GMYC maximum likelihood clusters or putative molecular species are reported in red, and by dark grey vertical blocks; vertical black blocks indicate the identified morphospecies (M); putative molecular species identified by bGMYC are represented by vertical solid colored boxes, colors indicate support values of Bayesian posterior probability (BPP): 0.5 – 0.9 in orange, 0.9 – 0.95 in ochre, 0.95 – 0.99 in yellow; light grey texture boxes indicate putative molecular species identified by ABGD. On the main nodes of the phylogram are reported the values of BPP ≥ 0.7; * = bpp < 0.7.

Figure 3. Species delimitation analysis based on *ITS2* gene sequences. A Bayesian ultrametric tree inferred from the *ITS2* gene sequence dataset, after the removal of identical haplotypes. *T. beneficus – T. sinensis* specimens are highlighted with different colors as follow: green = *T. sinensis*, from the present study; yellow = *T. sinensis,* from GenBank (Table 1); light blue = late-spring *T. beneficus* ecotype, from GenBank (Table 1); fuchsia = early-spring *T. beneficus* ecotype, from GenBank (Table 1).

Clades corresponding to GMYC maximum likelihood clusters or putative molecular species are reported in red, and by dark grey vertical blocks; vertical black blocks indicate the identified morphospecies (M); putative molecular species identified by bGMYC are represented by vertical solid colored boxes, colors indicate support values of Bayesian posterior probability (BPP): 0.5 – 0.9 in orange, 0.95 – 0.99 in yellow, 0.99 – 1 in white; light grey texture boxes indicate putative molecular species identified by ABGD. On the main nodes of the phylogram are reported the values of BPP ≥ 0.7.

Table 1. Samples analyzed in the study.

Species	♀♀	♂♂	*N**	Host gall wasp	Emergence	Gall provenience[a]	Vouchers/Accession no.	Marker	Reference
T. sinensis	5	6	11	*D. kuriphilus*	April 2006	IT - Piedmont Region	TsP06_1-11	*cox1*, ITS2	This study
"	5	3	8	"	May 2008	JP - Ibaraki Prefecture	TsJ08_1-8	"	"
"	3	3	6	"	May 2008	IT - Piedmont Region	TsP08_1-6	"	"
"	0	3	3	*B. pallida*	May 2013	"	TsPBp13_1-3	"	"
"	4	8	12	*D. kuriphilus*	March 2014	"	TsPd14_1-12	"	"
"	8	5	13	"	April 2014	"	TsP14_1-9, 11-14	"	"
"	7	4	11	"	April 2015	"	TsP15_1-11	"	"
"	7	8	15	"	April 2016	"	TsP16_1-15	"	"
T. affinis	2	0	2	"	March 2010	"	Taf10_1-2	"	"
"	3	0	3	*B. pallida*	March 2014	IT - Aosta Valley Region	Taf14_1-3	"	"
"	0	1	1	"	October 2014	"	TafA14_1	"	"
T. auratus	0	2	2	"	April 2014	IT - Liguria Region	Tau14_1-2	"	"
T. cyaneus	1	1	2	*D. kuriphilus*	May 2010	IT - Piedmont Region	Tc10_1-2	"	"
"	1	1	2	*C. divisa*	October 2014	"	Tc14_1-2	"	"
T. flavipes	0	3	3	*D. kuriphilus*	June 2014	IT - Aosta Valley Region	Tf14_1-3	"	"
T. geranii	1	0	1	"	August 2012	IT - Liguria Region	Tg12C_1	"	"
"	0	1	1	*C. quercusfolii*	April 2015	IT - Piedmont Region	Tg15Q_1	"	"
T. affinis	-	-	1	Oak cynipid	-	HU - Vas County	HM574341	*cox1*	Kaartinen et al. 2010
T. auratus	-	-	1	"	-	HU - Jász-Nagykun-Szolnok County	HM574340	"	"
T. beneficus late spring	-	-	4	*D. kuriphilus*	April 1988	JP - Ibaraki Prefecture	AB070476-AB070479	"	Yara 2004
T. beneficus early spring	-	-	4	"	March 1991	JP - Fukushima Prefecture	AB070473-AB070475, AB070480	"	"
"	-	-	4	"	March–April 1988	JP - Ibaraki Prefecture	AB070493 -AB070496	"	"
"	-	-	2	"	April 1993, 1997	JP - Nagano Prefecture	AB070497 - AB070498	"	"
"	-	-	1	"	March 1991	JP - Hiroshima Prefecture	AB070499	"	"
"	-	-	3	"	March–April 1993	JP - Kumamoto Prefecture	AB070501 - AB070503	"	"
"	-	-	1	"	March 1993	JP - Aomori Prefecture	AB070504	"	"
T. cyaneus	-	-	1	Oak cynipid	-	FI - Varsinais-Suomi Region	HM574245	"	Kaartinen et al. 2010
T. flavipes	-	-	1	-	-	SP	JQ416941	"	Stone et al. 2012
T. geranii	-	-	1	Oak cynipid	-	HU - Veszprém County	HM574309	"	Kaartinen et al. 2010
T. sinensis	-	-	4	*D. kuriphilus*	March–April 1993	CN - Hebei Province	AB070482 - AB070483, AB070485 - AB070486	"	Yara 2004
"	-	-	2	"	April 1993	CN - Liaoning Province	AB070484, AB070487	"	"
"	-	-	1	"	April 1992	KR - Kyongsangnam-do Province	AB070488	"	"
"	-	-	3	"	April 2000	KR - Kangwon-do Province	AB070489 - AB070491	"	"
"	-	-	1	"	April 1996	JP - Shimane Prefecture, Oki Islands	AB070492	"	"

Torymus sp.	-	-	1	*Celticecis japonica*	May 2000	JP - Chiba Prefecture	AB070500	"	"
"	-	-	1	*D. kuriphilus*	April 1993	JP - Aomori Prefecture	AB070481	"	"
T. flavipes	-	-	1	Oak cynipid		FI - Varsinais-Suomi Region	HM574237	ITS2	Kaartinen et al. 2010
T. geranii	1	0	1	*D. kuriphilus*	1997	JP - Ibaraki Prefecture	AB200280	"	Yara 2006
T. beneficus early spring	1	0	1	"	1988	JP - Ibaraki Prefecture	AB200271	"	"
T. beneficus late spring	1	0	1	"	1988	JP - Ibaraki Prefecture	AB200272	"	"
T. sinensis	-	-	1	"	1993	CN - Hebei Province	AB200274	"	"
Megastigmus dorsalis	-	-	1	-	-	CN - Guizhou	AY317240	*cox1*	Chen et al. 2004
"	-	-	1	Oak cynipid	-	-	GU123292	ITS2	Nicholls et al. 2010

Note. [a]: Country abbreviations according with Country codes ISO 3166.

Table 2. Frequency of genotype, expressed as number of individuals, for ITS2 sequences of *T. sinensis*.

Species/Collecting locality	Genotype		
	-4/-4 + G ((CT3, (AG3), (G)1).	-2/-2 ((CT)4, (AG)3)	0/0 ((CT)4, (AG)4)
T. sinensis (IT - – Piedmont)	1	13	50
T. sinensis (JP - – Ibaraki Prefecture)	0	1	7

Table 3. Within and between mean values of nucleotides *p*-distance[a].

***Torymus* species**	***p*-distance**								
	T. sinensis	*T. beneficus* LS	*T. beneficus* ES	*T. beneficus**	*T. auratus*	*T. geranii*	*T. affinis*	*T. cyaneus*	*T. flavipes*
T. sinensis	**1.3(0.3)/0.6(0.1)**	*1.8(0.4)*	*5.7(0.9)*	*4.8 (0.7)*	*10.1(1.3)*	*9.9(1.3)*	*11.4(1.3)*	*12.3(1.5)*	*12.1(1.5)*
T. beneficus LS	0.5(0.2)	**0.9(0.2)/-**	*5.2(0.6)*		*10.5(1.2)*	*25(1.8)*	*11.9(1.3)*	*21.9(2.1)*	*19.4(2)*
T. beneficus ES	0.5(0.2)	0	**0.3(0.1)/-**		*11.2(1.3)*	*24.9(1.8)*	*11.7(1.3)*	*21.9(2.1)*	*19.6(2)*
*T. beneficus**	0.5(0.2)			**2.3(0.2)/0**	*11.1(1.2)*	*25(1.8)*	*11.8(1.2)*	*21.9(2)*	*19.5(2)*
T. auratus	24.2(2.2)	23.5(2.1)	23.3(2.1)	23.4 (2.2)	**1.1(0.4)/0**	*11.6(1)*	*12.1(1.4)*	*29.9(2.6)*	*8.5(1.4)*
T. geranii	25.9(1.8)	10.8(1.3)	11.2(1.3)	11.1 (1.3)	9.7(1.3)	**0.7(0.3)/21(1.7)**	*12 1.4)*	*12.2(1.4)*	*13(1.5)*
T. affinis	24.8(2.1)	24.4(2)	24(2)	24.3 (2)	16.8(1.9)	19.5(1.6)	**1.6(0.4)/2.4(0.5)**	*29(2.3)*	*15.2(1.8)*
T. cyaneus	22.5(2.1)	12.7(1.5)	12.6(1.4)	12.6 (1.4)	13.1(1.5)	30.6(2.1)	11.5(1.4)	**0.1(0.1)/0.1(0.1)**	*25.3(2.5)*
T. flavipes	20.4(2.1)	11.8(1.3)	12.1(1.3)	12 (1.3)	10.4(1.4)	14.9(1.4)	12.5(1.5)	11.5(1.4)	**0.3(0.2)/1.2(0.4)**

[a]Number of base differences per site expressed as percentage. Above the diagonal are reported the mean values (italics) of between-taxa *p*-distance calculated on *cox1* gene; below the diagonal are reported the mean values of between-taxa *p*-distance calculated on ITS2; on the diagonal, in bold, are reported mean values of within-taxa *p*-distance calculated on *cox1* gene on ITS2, respectively on the right and on the left of /; standard deviation calculated on the base of 500 bootstrap is reported within brackets. *P*-distance values are calculated on nucleotide sequence databases without the removal of identical haplotypes.

[*]*p*-distance is calculated grouping sequences from *T. beneficus* early-spring (ES) and *T. beneficus* late-spring (LS).