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Integrative taxonomy of *Torymus* Dalman and *Ormyrus* Westwood species  
(Hymenoptera: Torymidae, Ormyridae) associated with cynipid gall wasps  
(Hymenoptera: Cynipidae)

## PHD THESIS SUMMARY

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## General introduction

As part of a biological control program directed against the invasive chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu implemented at the Institute of Agronomic Research (France, Nice), *Torymus sinensis* Kamijo was introduced to suppress the pest. The main activity carried out in France was to document the possible unwanted negative effects on the native community of *Torymus* as a result of the introduction of an exotic species. During the characterization of the parasitoid complex of *D. kuriphilus* with genetic and morphological approaches, attention was also directed to the genus *Ormyrus*. With only two reported species, *Ormyrus pomaceus* (Geoffroy) and *O. nitidulus* (Fabricius), molecular analyses showed greater diversity in terms of the number of species.

For the study of these taxa, the doctoral thesis was divided into two large chapters. The first part deals with the biological control agent *T. sinensis* in France, its population analysis and its relations with indigenous *Torymus* communities; for the first time this species was included in an identification key with focus on Europe. In the second part, the main topic is that of specific diversity of the genus *Ormyrus*, but with emphasis on the species that parasite the cynipid gall wasp on oaks and chestnut.

The main objectives of the doctoral thesis are:

O.I.a. Biometric characterization of the specimens from the "*beneficus-sinensis*" complex;

O.I.b Detection of molecular signals of historical hybridization within the complex;

O.I.c Documentation of the molecular diversity of the "*beneficus-sinensis*" complex in Europe;

O.II.a Delimitation of *Ormyrus* species and establishment of phylogenetic relationships between them;

O.II.b The integrative characterization of specimens of the genus *Ormyrus* obtained and their comparison with the types in the collection of the Museum of Natural History in Vienna, Austria and in Paris, France.

In order to achieve the objectives in this thesis, we have carried out the research activities both within the Research Group in Invertebrate Diversity and Phylogenetics in Faculty of

Biology (UAIC) and within the RDLB Team - Research and Development in Biological Control, the Institute of Agronomic Research in Sophia Antipolis.

# **1. Integrative taxonomy of *Torymus* Dalman species (Hymenoptera: Torymidae) associated with the invasive cynipid *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae)**

## **Introduction**

Originally from China, *Dryocosmus kuriphilus* was first accidentally introduced to Japan in 1941, where it became a major pest of chestnut trees (Murakami, 1997). This pest was then detected in Korea in 1958 (Cho and Lee, 1963), in North America in 1974 (Payne et al., 1976), in Nepal in 1999 (Aebi et al., 2006) and in Europe (Italy) in 2002 (Brussino et al., 2002). About 15 years after its first reporting in Europe, this pest has spread to areas with *Castanea sativa* Miller in almost all of Europe, colonising 25 countries (EPPO, 2007; Avtzis et al., 2018).

For the control of the invasive population in Japan, in 1975 a biological control program was implemented using *Torymus sinensis* as an auxiliary, with a visible control 8-10 years after its introduction (Murakami et al., 1977; Ôtake et al., 1984; Moriya et al., 2003). Following this success, *T. sinensis* was then introduced in the U.S. (Rieske, 2007), Italy (Quacchia et al., 2008), France (Borowiec et al., 2014), Slovenia, Croatia and Hungary (Matošević et al., 2015), as well as more recently in Portugal (A. Bento, personal communication), Spain (Nieves-Aldrey et al., 2019) and the Asian part of Turkey (Doğanlar and Senan, 2018).

Shortly after the introduction of the auxiliary *T. sinensis* in Japan, hybrid individuals were identified between it and *T. beneficus* Yasumatsu and Kamijo, an indigenous parasitoid with very close morphological and ecological features. At the same time, two strains have been identified for *T. beneficus*: early (TbE) and late (TbL) strains, having a one-month difference between the peaks of emergence. Males cannot be separated on the basis of morphological characters, and only the length of the ovipositor appears to differentiate females of *T. sinensis* from *T. beneficus* (Ôtake, 1987; Aoto and Murakami, 1992). With a shorter ovipositor and lower prolificity (Moriya et al., 1992), *T. beneficus* was replaced by *T. sinensis* by competition and introgression in the areas where *D. kuriphilus* is present (Yara et al., 2007). Individuals of *T. beneficus* are rare nowadays, recently, only one female and one male were found parasitizing *Dryocosmus murakamii* Ide and Abe (Matsuo et al., 2021).

Nine native species of *Torymus* which attacks *D. kuriphilus* have been reported in Europe: *T. auratus* (Müller) (Speranza et al., 2009; Muru et al., 2020; Kos et al., 2015), *T. erucarum* (Schrank) (Speranza et al., 2009), *T. fastuosus* Boheman (Kos et al., 2021), *T. flavipes* (Walker) (Speranza et al., 2009; Aebi et al., 2007; Kos et al., 2015), *T. nobilis* Boheman (Kos et al., 2021), *T. notatus* (Walker) (Gil-Tapetado et al., 2021), *T. formosus* (Walker) (Kos et al., 2015), *T. geranii* (Walker) (Kos et al., 2015), *T. scutellaris* (Walker) (Quacchia et al. 2012). However, no hybrid individuals have been identified in nature, but moreover, no laboratory reproductive compatibility has been observed between *T. sinensis* and the few native species of *Torymus* tested (Ferracini et al., 2017; Quacchia et al., 2014).

## **1.1. Genetic and morphological diversity in *Torymus sinensis* Kamijo**

### **1.1.1 Introduction**

The results of this part dealing with the morphological and molecular diversity of the biological control agent *Torymus sinensis* have already been published in collaboration with researchers from Sophia Agrobiotech Institute, Nice, France; Laboratory for Bio-safety, Chinese Academy of Agricultural Sciences, Beijing, China; Laboratory of Soil Biodiversity, Neuchâtel, Switzerland; Research Group in Invertebrate Diversity and Phylogenetics UAIC Iasi, Romania (Viciriuc et al., 2021).

### **1.1.2. Material and methods**

#### **Material biologic**

The specimens originated from three geographical regions: (i) China, the native area for both *D. kuriphilus* and *T. sinensis*, (ii) Japan, the first translocation area for *T. sinensis* and the native area for *T. beneficus*, and (iii) Europe (Italy and France), the second translocation zone. Individuals of *T. sinensis* from China were collected on *D. kuriphilus* from Hebei on *Castanea mollissima* Blume and Japanese individuals of *T. sinensis* in Tsukuba on *Castanea crenata* Sieb. and Zucc. Italian specimens were collected in three locations (Piedmont, Sardinia and Tuscany) on *Castanea sativa* Miller. Most of the French specimens were collected on *D. kuriphilus* under the classical biological control programme covering 69 sites (most of the area of spread of cultivated and natural chestnut trees in France) with a multi-annual sampling (2011–2018) (Borowiec et al., 2018 for details).

### **Collection of molecular data**

In this study, four molecular markers were used: (i) the mitochondrial protein-coding gene Cytochrome c oxidase subunit I (COI) with two overlapping regions amplified with the primers LCO1490/HCO2198 (Folmer et al. 1994) and COI pF2/COI 2437d (Simon et al., 1994; Kaartinen et al. (2010); (ii) the nuclear protein-coding gene Wingless (Wg), (iii) the nuclear protein-coding gene Enolase (Eno), which includes two introns (variable regions) and three exons (conserved regions); (iv) the ribosomal internal transcribed spacer 2 (ITS2).

Based on the COI dataset, we have implemented a series of tests based on an alignment of 413 base pairs such as: standard genetic parameters of the population, the number of haplotypes (h), diversity of haplotypes (Hd), diversity of nucleotides ( $\pi$ ), number of polymorphic sites (S) and total number of mutations (Eta), Tajima's D test (Tajima, 1989) and Fu's Fs (Fu, 1997). Using the frequency of haplotypes, we analyzed the molecular variance (AMOVA) with 10,000 permutations.

### **Morphological characterization**

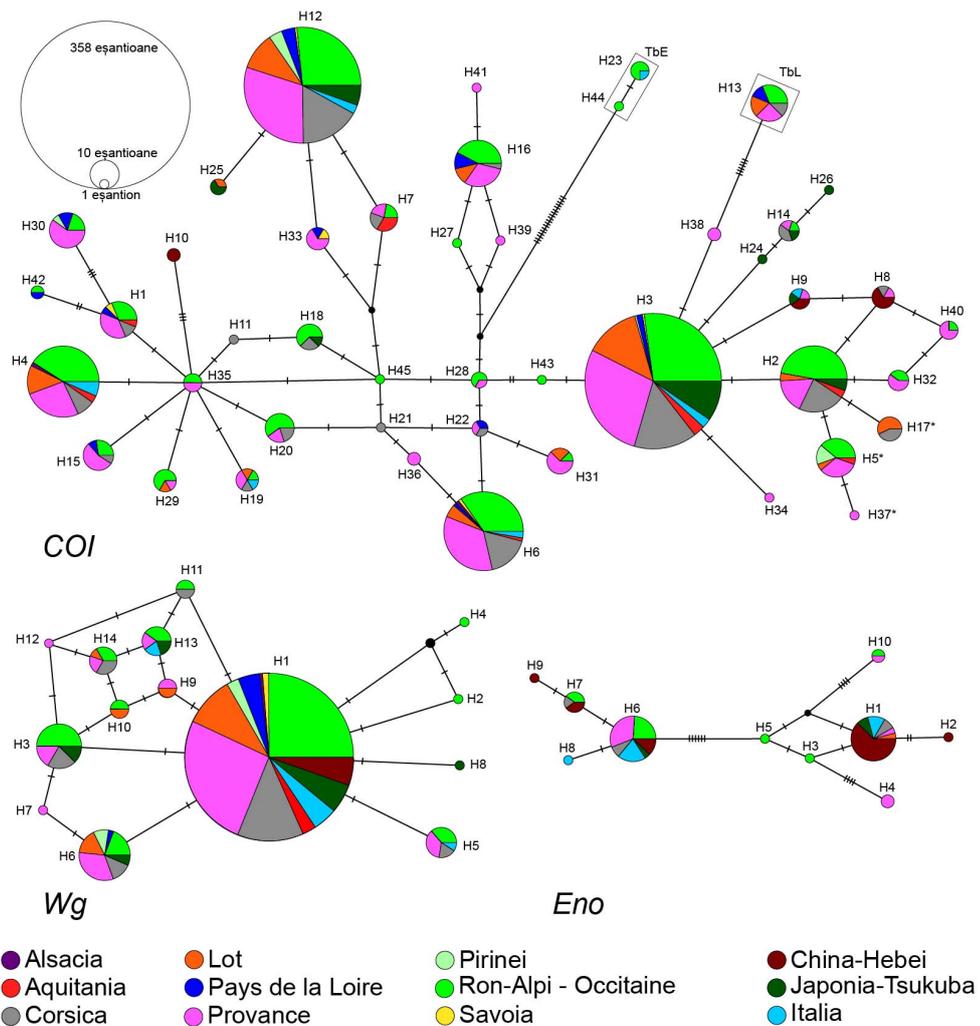
For morphometric analysis we selected 46 females and 45 males for which we measured 19 and 23 characters, respectively. For *T. beneficus* we measured only paratypes, four females and four males from the Entomology Laboratory, Ehime University (Japan). The observations and measurements of the morphological characters were made either using the Keyence VHX-2000 digital microscope or the Leica M205C microscope coupled with a Leica Application Suite v.4.0 image processing software (Leica microsystems GmbH). The data were analyzed using the multivariate ratio analysis (MRA) using the script for the R program published by Baur and Leuenberger (2011).

## **1.1.3. Results**

### **Diversity of COI haplotypes**

Based on the COI dataset for 796 individuals, we identified 45 haplotypes (Figure 1). Phylogenetic analyses led to the observation of two monophyletic molecular clusters. The first cluster contains the haplotypes H23 and H44 and the sequences of *T. beneficus* TbE submitted by Yara (2006) to GenBank. The second cluster groups the sequences obtained in this study with those of *T. sinensis* and *T. beneficus* TbL in Yara (2006).

In France, 0.54% of the individuals of *T. sinensis* have mitochondria of *T. beneficus* early strains (TbE) and 2.19% have mitochondria of *T. beneficus* late strains (TbL). The *p*-distance between molecular clusters is 5.3% for *T. sinensis* – *T. beneficus* TbE and 2.5% for *T. sinensis* – *T. beneficus* TbL. French regions with the highest number of haplotypes are Provence (31 haplotypes), Ron-Alps – Occitanie (28 haplotypes) and Corsica (19 haplotypes).



**Figure 1.** Haplotype networks generated on the basis of three genetic markers: COI, Wg and Eno. The three networks contain haplotypes present in France, Italy, China and Japan. The colors indicate haplotypes from different French counties or regions. For each haplotype the size of the circle is proportional to the observed frequencies. H – haplotype; H\* – haplotypes with an amino acid substitution. TbL – haplotypes derived from the late ecotype of *T. beneficus*; TbE – haplotypes derived from the early ecotype of *T. beneficus*.

In Italy, the diversity of haplotypes is large and similar to France,  $H_d = 0.85$  when haplotypes introduced by *T. beneficus* TbE are included, or  $H_d = 0.831$  when they are excluded. The investigated populations in China ( $H_d = 0.714$ ,  $\pi = 0.009$ ) and Japan ( $H_d = 0.679$ ,  $\pi = 0.01$ ) have a lower diversity compared to the European ones.

The results of Tajima's D and Fu's Fs tests performed using the COI sequences were negative in several cases, indicating an excess of rare alleles that could result from a rapidly expanding population after a genetic bottleneck. AMOVA analyses based on haplotype frequencies show that most genetic variability is found within the population (99.1%/99.11%) and much less between populations (0.9%/0.89%).

### **Diversity of ITS2**

Of the 46 individuals of *T. sinensis* that were sequenced for ITS2 in this study, 40 have the genotype 0/0, five the genotype -2/-2, and one individual has a genotype different from all those already reported by Yara (2006), which corresponds to (CT<sub>5</sub>)-(AG<sub>3</sub>), called 0\*/0\* (basically it has one more CT and one AG less than the 0/0 genotype).

### **The diversity of Wg and Eno**

Using the Wg sequences obtained from 283 individuals, we identified 14 haplotypes (Figure 1). The most common haplotype is H1, followed by H3 and H6. H1 is distributed in all areas included in the study, while H3 and H6 were detected only in France and Japan.

Following the analyses performed on 44 individuals, we identified 10 haplotypes on Eno (Figure 1). Haplotypes H1 and H6 are the most represented and have been found in all areas studied (China, Japan, Italy and France); both common haplotypes have the same number of connections. We have not detected divergent haplotypes that could indicate introgression.

### **Multilocus phylogeny**

In the multilocus phylogenies obtained, individuals of *T. sinensis* with mitochondrial DNA of *T. beneficus* TbE no longer form a distinct molecular clad, being instead included inside the main molecular cluster *T. sinensis*.

### **Analysis of the multivariate of their ratio (MRA)**

Morphological results showed a strong overlap for both females and males. Individuals of *T. sinensis* from France showed a high morphological variability. Only the female paratypes of *T. beneficus* and *T. sinensis* from China are well separated and can be differentiated using, the ratio between the second funicular segment *versus* the length of the metabasitarsus and the ratio between the ovipositor *versus* the length of the mesosoma. For males, the ratio between the malar space *versus* the length of the first funicular segment and the length of the head *versus* the length of the second funicular segment can be used.

## 1.1.4. Discussions

### Genetic variability in *T. sinensis*

According to Ôtake et al. (1984), a large number of *T. sinensis* specimens were introduced from China in Japan, and the introduction program in Italy was made using 2117 specimens imported from Japan (Quacchia et al., 2008). This should have been the unique import of *T. sinensis* from Asia and these individuals are thus the founders of European populations. The  $D$  values in the Tajima's test and  $F$  in the Fu's test, in several cases are negative and may be an indication of a past genetic bottleneck of the population followed by its recovery. However, for the introduction to Europe, *T. sinensis* went through at least three events in which bottlenecks must have occurred: during introductions to Japan from China, to Italy from Japan and to France from Italy.

The genetic diversity in the populations of *T. sinensis* increases the likelihood of the success of the biological control against the pest *D. kuriphilus* because a genetically diverse population is more likely to suppress the pest's population in the long term. In France, the greatest diversity of haplotypes and the greatest nucleotide diversity were recorded in the Pays de la Loire ( $Hd = 0.9$ ,  $\pi = 0.017$ ); the lowest values of haplotype diversity were recorded in the Pyrenees ( $Hd = 0.673$ ) and the lowest values of nucleotide diversity in Corsica and Aquitaine ( $\pi=0.01$ ). The diversity of the analyzed population in China is low,  $Hd = 0.714$  and  $\pi = 0.009$ .

The results of the AMOVA test show a lack of genetic differentiation between French populations, but this was to be expected because the introduced individuals are part of the same stock (Italian).

### Identity of *T. beneficus* TbL and TbE

The hypothesis is that *T. beneficus* TbL is part of *T. sinensis*, being simply individuals with short ovipositor. They were classified as such because of the large variability of the length of ovipositor sheaths in *T. sinensis*. In addition, longevity and the pre-oviposition period are similar for *T. beneficus* TbL and *T. sinensis*, except that *T. sinensis* is able to lay about four times as many eggs (Piao and Moriya, 1992). This indicates that *T. beneficus* TbL could be a distinct line of *T. sinensis* with shorter ovipositor and unknown origin; being less prolific and less effective (Piao and Moriya, 1992; Yara et al., 2012) was excluded by competition.

### **Evidence of hybridization**

No positive evidence for hybridization between *T. sinensis* and European *Torymus* species was found in this study. All sequences obtained in this study were consistent for the three nuclear (Eno, Wg and ITS2) and mitochondrial (COI) genes. The only exception was the detection of COI sequences corresponding to *T. beneficus* TbE in some specimens that based on the other nuclear genes and morphometric analysis are *T. sinensis*. However, hybridization occurred before the introduction of *T. sinensis* in Europe.

### **Distinctive characters between *T. beneficus* and *T. sinensis***

Morphometric analysis failed to separate the paratypes of *T. beneficus* from European individuals of *T. sinensis* or specimens of *T. sinensis* with haplotypes of *T. beneficus* TbE due to the high variability of specimens of *T. sinensis*. Paratypes of *T. beneficus* are well separated in the PCA analysis from *T. sinensis* collected in China, and individuals of *T. sinensis* collected in Japan and France are positioned in between. The length of the ovipositor used by Kamijo (1982) and later authors to separate *T. beneficus* from *T. sinensis* is discriminatory only if European *T. sinensis* is not taken into account because variability in these populations was high. The only difference between the two species is quantitative and relates to the length of the antennae (Kamijo, 1982; Viciriuc et al., 2021).

## **1.2. *Torymus sinensis* and nearby native species**

### **1.2.1. Introduction**

To date, no reports of hybridization between *T. sinensis* and any European *Torymus* have been observed for individuals collected in the field. Even though no signs of hybridization with native species have been identified, further research has been carried out. This study is a continuation of the previous one in which the aim is to identify native parasitoids of the genus *Torymus* that attack *Driocosmus kuriphilus* in France and to find the closest native species that could be candidates for intraspecific hybridization. Previous studies have been based on two genes that are commonly used in species delimitation, namely COI and ITS2 (Yara, 2004; Yara, 2006), and the goal is to also add other markers used in phylogenetic reconstructions. I also propose an identification key to facilitate the identification of the native species of *Torymus* reported so far on *D. kuriphilus* throughout Europe, in order to support the monitoring programs for the chestnut gall wasp and its parasitoids.

## 1.2.2. Materials and methods

### Biological material

Various cynipid gall wasps were collected on species of the genus *Quercus* and *Rosa*, such as: *Biorhiza pallida* (Olivier) sexual generation, *Cynips longiventris* Hartig asexual generation and *Diplolepis rosae* (Linnaeus) in France, in ten locations: Aynac, Campile, Conteville, Fozières, La-Garde-Freinet, Lutterbach, Malmerspach, Rignac, Rians and Saint-Cassien. In Romania, galls were collected from two sites in Botoşani and Iaşi. Most of the native French parasitoids were obtained from *D. kuriphilus* on *Castanea sativa* as part of the French biological control program from which individuals of *Torymus sinensis* were previously obtained.

### Molecular characterization

Similar to the previous study, in this study four molecular markers were used: (i) the mitochondrial protein-coding gene Cytochrome c oxidase subunit I (COI); (ii) the nuclear protein-coding gene Wingless (Wg), (iii) the nuclear protein-coding gene Enolase (Eno); (iv) the ribosomal internal transcribed spacer 2 (ITS2).

### Analysis of molecular data

For all markers, phylogenetic reconstructions were performed using the public resource for the inference of large phylogenetic trees, CIPRES Science Gateway v.3.1. At the same time, the dataset was concatenated and analyzed with both a method of maximum likelihood (ML) and a method of Bayesian inference. The *p*-distances between taxa were calculated for all molecular markers using uniform rates between sites and the elimination of missing data between pairs of sequences.

### Morphological characterization

#### Preparation and observation of specimens

After the extraction of the DNA, all specimens were dried using hexamethyldisilylase (HMDS; Cowan 1995). Observations and measurements of morphological characters were made similar to the previous study. We mainly used specimens of *Torymus* species collected on *D. kuriphilus* in France (*T. auratus*, *T. flavipes*, *T. notatus* and *T. sinensis*), as well as species collected on other cynipid gall wasp on oak (*T. affinis*, *T. cyaneus* and *T. geranii*). In the identification key we also included the species of *Torymus* that attack

*D. kuriphilus* in Europe: *T. fastuosus*, *T. nobilis*, *T. formosus*, *T. erucarum* and *T. scutellaris*. They come from Richard Robinson Askew personal collection (UK).

### **Data analysis**

For morphological analyses, the first step was to define an isometric axis of size (isosize), then perform a shape PCA (i.e. a PCA in the space of all ratios). We performed a PCA ratio spectrum and an allometry ratio spectrum to visualize the ratios that explain most of the variation and those that show the highest amount of allometry. Finally, we applied the LDA ratio extractor to obtain a list of reports that best discriminate against the groups previously identified after the shape PCA.

## **1.2.3. Results**

### **Native parasitoids**

We have identified four species of *Torymus* that attack *D. kuriphilus* in France: *T. auratus*, *T. flavipes*, *T. notatus*, and *T. rubi* (Schrank). For the first time *T. rubi* (a female) was reported on *D. kuriphilus*. Furthermore, during the collection of galas induced by indigenous cinipids to have better coverage of the fauna of *Torymus*, we identified a female of *T. sinensis* parasitizing *Biorhiza pallida* sexual generation.

### **Molecular results**

In order to establish phylogenetic relationships between *T. sinensis* and the native species, I used the sequences from the previous study for *T. sinensis*, *T. auratus* and *T. geranii*. For the same group of molecular markers (COI, Wingless, Enolase and ITS2) we have added sequences obtained from different phylogenetically close taxa (*T. notatus*, *T. cyaneus*, *T. rubi*, *T. flavipes*, *T. bedeguaris*), but also used as outgroup *Glyphomerus stigma*.

### **COI**

The phylogenetic reconstruction carried out allowed to discriminate all *Torymus* species previously identified using morphological characters. The *sinensis* cluster is divided into two, most individuals of *T. sinensis* and some of *T. sinensis* with mitochondrial DNA of *T. beneficus* (TbE). The *sinensis-beneficus* cluster is sister to one containing *T. notatus*, *T. cyaneus* and *T. rubi*. The genetic *p*-distance between the sequences of *Torymus sinensis* and those derived from *T. beneficus* is relatively small, only 5.8%. The native species that are closest to *T. sinensis* are *T. rubi* (9.9%), *T. geranii* (10.3%) and *T. auratus* (10.4%).

### **Wingless**

In the phylogeny based on information from this nuclear-coding gene, Wingless, the *T. sinensis* cluster is not divided and is sister to *T. cyaneus*, and the two form a monofilum with *T. notatus*. This genetic marker cannot discriminate between very close species such as *T. auratus* and *T. geranii*. *Torymus notatus* (2%) and *T. cyaneus* (3.7%) are the native species closest to *T. sinensis* based on this marker.

### **Enolase**

In the phylogeny based on Enolase, *T. sinensis* cluster is the sister to *T. notatus*, and the two species are further grouped with *T. cyaneus*. The native species closest to *T. sinensis* are *T. notatus* with a *p*-value of 1.5% followed by *T. geranii* and *T. auratus* with 3.3% and 3.7%, respectively.

### **ITS2**

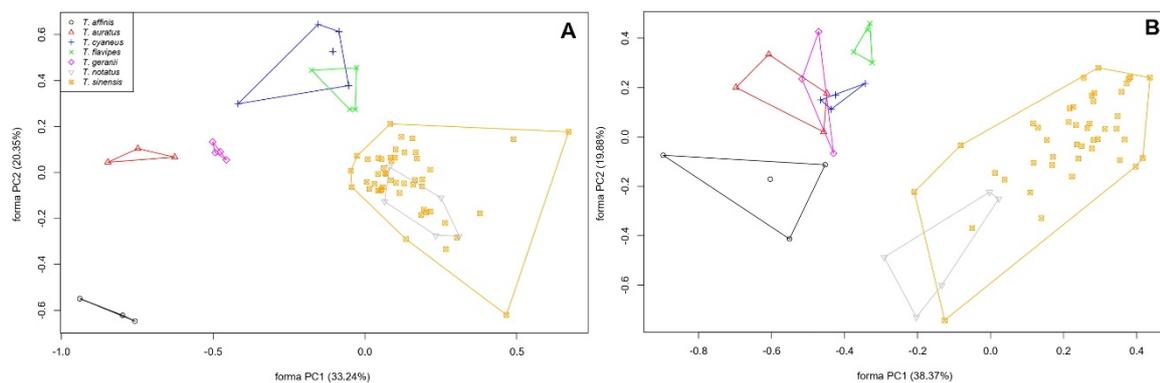
Using the information provided by the sequences of ITS2, the cluster containing *T. sinensis* is sister to *T. notatus*. *Torymus cyaneus* is no longer close to them, grouping with *T. auratus* and *T. gerani* (these are again distinct), *T. rubi*, *T. bedeguaris* and *T. flavipes*. Also, the closest species to *T. sinensis* are *T. notatus* and *T. cyaneus* with a *p*-distance of 14.1% and 17.2%, respectively.

### **Multi-locus phylogeny for *T. sinensis* and allied species**

After concatenation, numerous evolutionary models and various partitioning schemes were tested. Phylogenetic analyses unambiguously discriminate against European *Torymus* species as well as the "*beneficus-sinensis*" complex, providing a reliable basis for species delimitation, morphological characterization and identification of diagnostic characters. *Torymus notatus* and *T. cyaneus* are the species closest phylogenetically with the "*beneficus-sinensis*" complex.

### **Morphometric results**

As a result of the shape PCA analysis, for both females and males, the first component is the most discriminatory; all native species can be discriminated from *T. sinensis* along this axis (Figures 2 A female and 2 B Male), with the exception of *T. notatus* showing a strong overlap with *T. sinensis*. For both sexes, the axis scatterplot of isosize compared to first shape PC indicates that *T. sinensis* has the greatest variability in size and that this species is on average smaller than other native species, with the exception of *T. notatus*.



**Figure 2.** The results of multivariate ratio analysis (MRA) for the *Torymus* species used in this study. (A) the first component *versus* the second component for females; (B) the first component *versus* the second component for males.

### 1.2.3.1. The key to *Torymus* species parasitizing *Dryocosmus kuriphilus* in Europe

In order to build an identification key for use in Europe, we have included *Torymus* species obtained from gales of *D. kuriphilus* in France (*T. auratus*, *T. flavipes*, *T. notatus* and *T. sinensis*) and those identified in other European countries (*T. fastuosus*, *T. nobilis*, *T. formosus*, *T. erucarum* and *T. scutellaris*), as well as the species collected on other cynipid gall wasp on oak (*T. affinis*, *T. cyaneus* and *T. geranii*), but phylogenetically close to *T. sinensis*. The main characters included in the key are at the level of the antennae, head in dorsal view, mesoscutellum, mesopleuron, propodeum, gaster, hind leg and hind wing.

### 1.2.4. Discussions

Four native species of *Torymus* parasitizing *D. kuriphilus* have been identified so far in France: *T. auratus*, *T. flavipes*, *T. notatus* and *T. rubi*. At the same time, since the appearance in Europe of the chestnut gall wasp, four other native species than those detected in France have been reported in Europe: *T. fastuosus*, *T. erucarum*, *T. nobilis* and *T. scutellaris*. With the exception of *T. rubi*, first reported on this host, the others are common species for cynipid gall wasps on oak (Askew et al., 2013).

Phylogenetic and morphological data show that *T. notatus*, indigenous to Europe, reported in France (this study) and Spain (Gil-Tapetado et al., 2021) on *D. kuriphilus*, is most likely to hybridize with the biological control agent *T. sinensis*. In addition to the

morphological similarity, they also have the same flight period (Pogolotti et al., 2019). They share the same host, *D. kuriphilus*, but also other species of cynipids such as: *Andricus aries*, *A. curvator*, *A. cydoniae*, *A. inflator*, *A. kollari*, *A. lignicolus*, *A. polycerus* and *B. pallida*, where *T. sinensis* has been reported lately (Ferracini et al., 2017).

## **2. Integrative taxonomy of *Ormyrus* Westwood species (Hymenoptera: Ormyridae) associated with cynipids gall wasp on oaks**

### **2.1. Introduction**

The taxonomy of the Ormyridae family is not fully solved, even though Zerova and Seryogia (2006) identify 34 species for the Palearctic region. The study was conducted only on the basis of morphological characters, and an integrative approach is necessary. The presence of cryptic species for *Ormyrus pomaceus* (Geoffroy) and *O. nitidulus* (Fabricius) has been found by Kaartinen et al. (2010), the same being pointed out by José Luis Nieves-Aldrey (National Museum of Natural Sciences - Madrid, unpublished data).

#### **Genus *Ormyrus***

The *Ormyrus* species in the Palearctic region have been classified into two groups of species: the *diffinis* group (females without a median carina on the gaster) and the *orientalis* group (females with a median carina on the gaster) (Zerova and Seryogia, 2006). For the Palearctic region, Zerova and Seryogia (2006) record 34 species of *Ormyrus*, 23 of the *diffinis* species group and 11 of the *orientalis* group.

### **2.2. Materials and methods**

#### **Study area and collection of entomological material**

In order to obtain the entomological material, in the period 2011-2018 I collected a series of galls induced by different species of the tribe Cynipini. In addition to this method, specimens were collected equally with the sweep net and the Malaise trap. The study area is represented by: Romania (13 localities) France (8 localities), Italy, Bulgaria, Hungary and South Korea (one locality each).

### **DNA extraction and amplification**

For DNA extraction, we used a non-destructive method adapted for extraction from small insects to avoid their destruction, because the same specimens are also used for morphological analysis.

### **Reagents and conditions for PCR**

In order to amplify the standard region, the "DNA barcode" (Hebert et al., 2003) we used the primers: LCO1490 and HCO2198 developed by Folmer et al. (1998), and for some specimens where the sequencing reaction failed, we used the other COI primers COIpF2 and COI2437d (Simon et al., 1994; Kaartinen et al., 2010).

### **Delimitation of species by means of bioinformatic methods**

The phylogenetic reconstruction was carried out on the CIPRES SCIENCE GATEWAY portal using the RAxML– HPC2 XSEDE program (Stamatakis, 2014). The genetic *p*-distance between species groups and within species groups was calculated in the Mega 6.06 program (Kumar et al., 2018).

### **Morphological characterization of specimens**

Following DNA extraction, all specimens were dried using hexamethyldisalyzant (HMDS; Cowan, 1995). All adults were identified using the identification key proposed by Zerova and Seryogina (2006) for the Ormyridae fauna of the Palearctic region. Observations and measurements of morphological characters were made using the Leica S6 binocular stereomicroscope. The images were taken using the Leica M205C stereomicroscope coupled with a Leica Application Suite v.4.0 image processing software (Leica microsystems GmbH), composite images were produced with Zerene Stacker, and contrast and brightness were adjusted in Adobe Photoshop 2020.

## **2.3. Results**

### **Phylogenetic reconstruction**

In the phylogenetic reconstruction, three main molecular clusters were delimited, corresponding to the groups of species as follows: one cluster for the *Ormyrus diffinis* group and two other cluster corresponding to the *O. orientalis* group of species.

### **Delimitation of species based on molecular markers**

**The PTP algorithm** delineated 28 possible species with probability values between 0.081 and 1. Of these, 17 correspond to species of the *O. orientalis* group and 11 to *O. diffinis* group.

**Interspecific  $p$  distances.** The groups were made after delimitation using the PTP algorithm. The values for interspecific  $p$  distances fall within the range of 2.16%–20.93%. Intraspecific  $p$  distances within the identified groups are between 0.34% and 3.36%.

### **Morphological characterization**

Since the subject of this study is that of integrative taxonomy of species of the genus *Ormyrus* associated with cynipids gall wasp on oaks and chestnut, we have characterized from a morphological point of view only representatives from the *orientalis* group of species. Moreover, for each identified molecular species we chose 2 females to better capture the variability, and where the molecular clade was represented by few individuals, we chose only one female. We have not characterized morphologically representatives of molecular species where there were only males because until now the separation of species of this genus is done only on the basis of female characters and the holotypes are also only females.

We have made descriptions for 8 specimens of the type housed at the Museum of Natural History in Vienna, Austria, for comparison with the material held for identification.

## **2.4. Discussions**

### **Phylogenetic analyses**

Phylogenetic reconstruction delineates species groups of the genus *Ormyrus* with slightly different strategies, so the *O. diffinis* group is separated from the *O. orientalis* group. Species of *O. diffinis* group parasitize in galls induced by dipterans or cynipids on herbaceous plants, and those of *O. orientalis* group parasitize in addition to the hosts of *O. diffinis* group also arboreal cynipids gall wasp on oaks or chestnut trees (family Fagaceae).

For the Palearctic region, 11 species from the *orientalis* group were reported (Zerova and Seryogina, 2006) while in this study we identified 17 possible species. For the *O. diffinis* species group we have identified 11 possible species, Zerova and Seryogina (2006) report 23 species for the Palearctic region.

## **Morphological analyses**

Since the taxonomy of this group is not fully known, morphological identification was carried out using the last taxonomic revision of Zerova and Seryogina (2006). The only molecular group to which we could attribute a definite binomial name was molecular species 12, identified as *O. nitidulus*. Of the 8 type specimens from the Vienna collection, 5 (*O. aerosus* Förster, *O. blandus* Förster, *O. viridanus* Förster, *O. placidus* Förster and *O. prodigus* Förster) which are synonymous with *O. pomaceus*, at the first sight at least two synonyms, *O. aerosus* and *O. blandus* are most likely distinct species and have been wrongly synonymised.

### **2.5. *Ormyrus nitidulus* Fabricius case study**

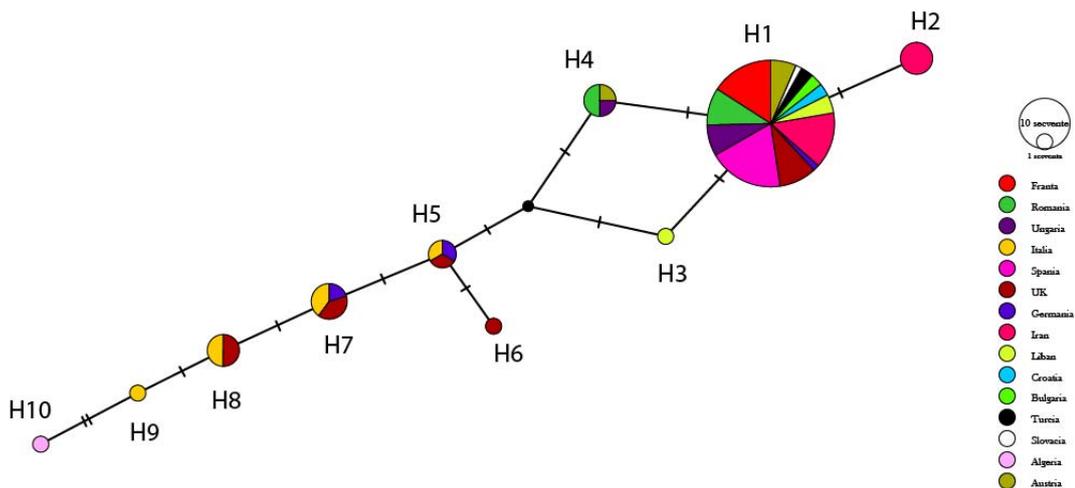
In order to build a haplotype network, we used the COI sequences from 93 individuals, and the selected region had a length of 544 base pairs. In addition, to confirm the relationship between individuals, we also used the nuclear marker ribosomal ITS2 (internal transcribed spacer 2), proposed by Zao et al. (2010) as an alternative marker for a "barcode". The number of sequences was 87, of which 24 were provided by Graham Stone, Professor of Ecology at the University of Edinburgh.

#### **Results using the COI marker**

For the mitochondrial dataset we identified 81 haplotypes with 88 variable sites and a total number of mutations 111 (Eta), haplotype diversity 0.993 (Hd) and nucleotide diversity 0.03594 ( $\pi$ ). Most haplotypes, 96%, are unique haplotypes with many connections, up to 18, and arranged towards the outside of the network. From the haplotypes network it is not possible to identify a dominant haplotype from which to derive other haplotypes. The haplotype network made with the sequences of the mitochondrial marker COI cannot reconstruct the migration routes of this species.

#### **Results using the ITS2 marker**

The nuclear dataset consisted of 87 sequences with lengths from 400 to 414 base pairs. 10 haplotypes (Figure 3) were identified with 10 variable sites and a haplotyped diversity of 0.470 (Hd). The total number of mutations being 10 (Eta) and the nucleotide diversity ( $\pi$ ) is 0.14943.



**Figure 3.** The haplotype network generated on the basis of the genetic marker ITS2. The colors indicate haplotypes from different countries. For each haplotype the size of the circle is proportional to the observed frequencies. H – haplotype.

Haplotype 1 is the most common and groups sequences from 63 individuals with distribution in all of the sampled countries, the only exceptions being Algeria and Italy. From H1 are derived other 3 haplotypes with lower frequency, of 1 and 4 individuals respectively. Haplotypes 2 and 3 have been identified only in Iran and Lebanon respectively. The countries with the highest diversity of haplotypes are the UK with a number of 5 haplotypes and Italy with 4 haplotypes.

### **Morphological characterization**

Morphological characterization for males of the species *Ormyrus nitidulus* is carried out for the first time. Since its identification was made using morphological characters for females it was possible to correlate conspecific male and female individuals using molecular data. After a thorough morphological characterization, morphological variations has also been identified that are closely dependent on the size of the individual, thus, for individuals with small sizes most variations are at the level of the antennae and color.

Both males and females show a high degree of morphological plasticity. Females also vary in size, implicitly leading to a variation in color, but also to differences in metasoma sculpture and differences in the antennae. In smaller specimens, the dark color predominates; the sculpture is less marked; at the level of the antenna, the setae on the flagellomeres are drastically reduced; the length of the flagellar segments is changed.

## General conclusions

On the basis of the COI sequences, we identified haplotypes belonging to the two strains of *Torymus beneficus* (early strain, TbE and late strain, TbL) in individuals of *T. sinensis* in Europe. The stock imported from Italy to France for the control of the invasive species *Dryocosmus kuriphilus* is *T. sinensis* mildly introgressed with both ecotypes of *T. beneficus*.

The results of the AMOVA test show the lack of genetic differentiation between the French populations, but this was to be expected because the introduced individuals of *T. sinensis* are part of the same stock (they were all imported from Italy).

Based on morphometric analyses, pure *T. sinensis* from China and specimens of the type series of *T. beneficus* from Japan can be separated on the basis of 4 ratios (for females: the length of the second funicular segment *versus* the length of the mesobasitarsus, the length of the ovipositor *versus* the length of the mesosoma; for males: malar space *versus* length of the first funicular segment, length of the head *versus* the length of the second funicular segment).

The native species of *Torymus* that have been identified parasitizing *D. kuriphilus* in France are *T. auratus*, *T. flavipes*, *T. notatus* and *T. rubi*. Of these, *T. rubi* represents the first record for the communities of *Torymus* installed on *D. kuriphilus*.

Molecular and morphological results confirm that *T. notatus* may be a candidate for interspecific hybridization with *T. sinensis*.

The specific diversity for the genus *Ormyrus* in the community of parasitoids in the galls of cynipid gall wasp was underestimated to only two species: *O. pomaceus* and *O. nitidulus*; in this study we identified 18 possible molecular species. Of these, 11 are associated with cynipid gall wasp on oaks and chestnut trees, 2 with cynipid gall wasp on herbaceous plants, and 5 have unknown biology, but are grouped in the same molecular cluster.

The mitochondrial COI marker can be used in the case of the genus *Ormyrus* for species delimitations, but it cannot restore the migration routes at least for *O. nitidulus*. The nuclear marker ITS2 can be used as an alternative for phylogeographic reconstructions, being the case for molecular species 12, identified as *O. nitidulus*.

Since the taxonomy of this group of species is uncertain and complicated, it was possible to identify with certainty, using morphological characters, only one molecular group (molecular species 12) as being *O. nitidulus*, at the same time this species has a high degree of morphological plasticity.

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