

**PHYLOGENY AND PHYLOGEOGRAPHY OF  
INQUILINE OAK GALLWASPS**

Ph.D. thesis

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Szeged  
2011

## Introduction

Gall induction has evolved many times in insects, which is assumed to be a special relationship between the insect and its host plant. Gall wasps (Hymenoptera: Cynipidae) comprise one of the most species-rich groups of gallers, capable of inducing a wide variety of structures on a wide range of host plants. Cynipid galls support a specific community of arthropods, including parasitoid natural enemies and herbivorous inquilines. Cynipid oak gall wasp communities have become a model system to study the community structure and evolution, because they are relatively closed and easy to sample.

Approximately 15% of the species of the Cynipidae family - comprising the inquiline tribe Synergini - have lost the ability to induce galls but are obligate inhabitants of galls induced by other species. These cynipid inquilines are able to modify the differentiation of host plant tissues to form larval chambers within which they feed on specialized nutritive gall tissue. Of the 176 known species belonging to the tribe Synergini, 48 can be found in the Western Palearctic. The gall wasp fauna of the Carpathian Basin is particularly rich and sequence-based studies reported high within-species genetic diversity in a number of gall-inducing cynipid species.

Most of the studies dealing with gall wasps have focussed on gall inducers and their parasitoid enemies, therefore inquilines are the least known components of the gall wasp community, although they are an abundant and integral part of it. A number of important taxonomic and phylogenetic issues remain unresolved for cynipid inquilines, and in order to address them, molecular phylogenetic analyses may be needed beside the morphological re-appraisal of diagnostic species-level characters. Previous works on gall wasps have shown that a fragment of the nuclear 28S D2 expansion segment is applicable for species level differentiation, while the mitochondrial cytochrome oxidase I (COI) and cytochrome b (cytb) genes are useful for within species studies.

One of the inquiline groups we have studied in detail is the genus *Synophrus* (Hymenoptera: Cynipidae: Synergini), which develop on *Quercus cerris* and is notable in many respects. *Synophrus* species attack host galls at a very early

stage of gall development, grow faster than the host larva, which is finally eliminated because of food deprivation and is therefore unknown. *Synophrus* larvae 'hijack' gall development and cause species-specific gall morphology, which is unusual among inquilines. However, there is significant diversity in the morphology, size and location of *Synophrus* galls. Prior to our study 3 *Synophrus* species were recognized in the Western Palearctic: *S. pilulae* Houard, 1911, *S. politus* Hartig, 1843, and *S. olivieri* Kieffer, 1898.

The other object of our study was the inquiline species *Synergus umbraculus* Olivier, 1791, which belongs to the largest and most problematic genus of the Synergini, the genus *Synergus*. This species is widely distributed in the Western Palearctic and has a wide range of cynipid hosts, but most frequently develops in the asexual galls of *Andricus* species (Hymenoptera: Cynipidae: Cynipini). Previously it was hypothesized that *S. umbraculus* is a complex of cryptic species. This was confirmed in a recent study focusing on the sequence-based phylogenetic reconstruction of the tribe Synergini. In this recent study it was demonstrated that, as currently recognized on the basis of morphology, *S. umbraculus* comprises four distinct molecular taxa (MOTUs). In the present study, we examine a single monophyletic lineage that includes > 90% of the sequenced individuals, and refer to it as *S. umbraculus*.

## Objectives of the study

Regarding the genus *Synophrus* our main objective was to assess phylogenetic relationships within the genus using nuclear (28S D2) and mitochondrial (COI) sequence information.

Regarding *Synergus umbraculus*, our main aim was to analyze the genetic diversity of the *S. umbraculus* population of the Carpathian Basin, considering the effects of Quaternary glaciations and possible cynipid host-associated differentiation (HAD). Besides, we wanted to examine phylogeographic patterns across the Western Palearctic in an analysis incorporating samples from all putative southern glacial refugia.

## Methods

### Field collections

*Synophrus* containing cynipid galls were collected from *Quercus cerris* in four regions of Hungary: the Southwest (Mecsek mountains), the Northwest (Sopron mountains), the Northeast (Bükk mountains) and Southeast (Szeged, the Great Plain). The location on the plant was recorded and categorized. Other samples from different regions of the Western Palearctic were examined, as well.

We used *S. umbraculus* from 55 locations across the species' range from Spain to Iran, incorporating the putative Western Palearctic refugia and a wide range of cynipid gall wasp hosts. According to our aims, we collected galls from 18 locations in Hungary to allow examination of spatial patterns of genetic diversity and HAD. Wasps were reared from galls under quarantine and were stored in 96% ethanol at -20 °C until further analysis.

### DNA extraction, amplification, sequencing and assembly of sequences

Genomic DNA was extracted from 1-3 hind legs using DNeasy Tissue Kit (QIAGEN) in accordance with the manufacturer's instructions for insect DNA

extraction, or using a cheap but efficient chelex-based procedure. The remaining part of the adult body was retained for further morphological examination and for deposition of vouchers.

In the study of the genus *Synophrus* a 658 basepair (bp) fragment of the mitochondrial COI gene was amplified. A subset of individuals was also sequenced for the nuclear 28S D2 locus (565 bp). In the study of *Synergus umbraculus* a 697 bp fragment of the mitochondrial *cytb* gene was amplified for all individuals, while a 489 bp fragment of the 28S D2 locus and a 658 bp fragment of the COI gene were amplified for a subset of the samples. The purified PCR products were sequenced.

All gap positions of the *Synophrus* 28S D2 sequences were restricted to be congruent with previous work on cynipid gall wasps. Gaps in the aligned sequences were coded as 14 separate binary characters. Sequences of the following closely-related Synergini species were included in our analysis: *Saphonecrus haimi*, *Saphonecrus undulatus*, *Saphonecrus connatus*, *Saphonecrus barbotini*, *Saphonecrus lusitanicus* and *Rhoophilus loewi*.

### Sequence analysis

In the analysis of the genus *Synophrus* the GTR+I+G model (general time reversible model with gamma-distributed rates across sites and a proportion of invariant sites) was used for COI, and the SYM+I model (symmetrical model with a proportion of invariant sites) was used for 28S D2. Phylogenies were estimated in a Bayesian framework (MrBayes 3.1.2) in the following data partitioning schemes: COI, COI partitioned by codon position, 28S D2 with or without a separate gap partition, and COI–28S D2–28S D2 gap partition combined.

In the analysis of the *Synergus umbraculus* data full statistical analyses were performed only on the *cytb* sequences. Within-population variability was quantified as the total number of haplotypes, the number of haplotypes unique to a given sample, gene (*h*) and nucleotide (*p*) diversity, and with two types of genetic distances. The diversity of haplogroups was quantified with the same indices, as well.

After testing for the suitability of applying a tree based phylogenetic analysis, phylogenetic relationships among cytb haplotypes were estimated in a Bayesian framework (BEAST 1.4.8) using birth-death prior and applying a strict molecular clock. The GTR+I+G nucleotide substitution model was applied, which was partitioned across codon positions with parameters unlinked across partitions. We calibrated sequence divergence using a rate of 1.15% sequence divergence per million years per lineage. We generated a statistical parsimony network for a subset of haplogroups to resolve relationships among them.

Spatial genetic structure was investigated using analysis of molecular variance (AMOVA) with two types of genetic distances: the number of pairwise differences between sequences, and pairwise patristic distance. We also carried out AMOVA to test for cynipid host-associated differentiation.

## Results and discussion

### Genus *Synophrus*

- Based on our molecular phylogenetic results and subsequent re-appraisal of diagnostic morphological characters four additional new species have been established: *Synophrus hungaricus*, Melika and Mikó, *S. libani*, Melika and Pujade-Villar, *S. syriacus*, Melika and *S. hispanicus*, Pujade-Villar.
- Our dataset supports the monophyly of all Western Palearctic *Synophrus* species, but reject monophyly of the studied *Saphonecrus* species. The genus *Synophrus* and *Saphonecrus barbotini* together with *Sa. lusitanicus* are sister groups, while *Sa. connatus* and the *Sa. haimi* - *Sa. undulatus* species pair form a separate lineage. The affiliation of *Sa. connatus* remains unresolved.
- Two lineages come out as strongly supported within *Synophrus*, one comprising *S. hispanicus-hungaricus-olivieri* and the other comprising *S. pilulae-politus-libani-syriacus*. In the first lineage a Spanish (*S.*

*hispanicus*), a Central-European (*S. hungaricus*) and an Iranian/Turkish (*S. olivieri*) species can be found. Each species represents a distinct geographic region and they form species pairs. Similar geographic separation is present in the second lineage: *S. syriacus* (Iran and Syria) and *S. libani* (Lebanon) form a monophyletic group, which is the sister group of the more widely dispersed *S. politus* (Hungary, Italy, Turkey). *S. pilulae* (Central Europe) forms the sister group of these three species of the second lineage.

- The two lineages mentioned above can be differentiated on the basis of gall morphology, as well. Species of the first lineage (*S. hispanicus* - *hungaricus* – *olivieri*) emerge from irregular swellings in twigs. *S. olivieri* has multilocular galls. Species of the second lineage (*S. pilulae* – *politus* – *libani* – *syriacus*) emerge from more regularly spherical bud galls. The location of *S. politus* galls shows considerable variation. Because gall location is determined by the oviposition preference of the females of the host gall wasp species, this variation suggests either that host gall wasp species show occasional variation in preferred gall induction sites, or that *S. politus* attacks different host gall wasp species, inducing galls in different locations.

### ***Synergus umbraculus***

- Based on 239 cytb sequences 8 haplotype groups can be defined (haplogroups, H1-H8), of which 6 are present in the Carpathian Basin. COI sequences supported the same subdivision, while 28S D2 sequences showed low variabilities. We found the highest genetic diversity in the Carpathian Basin, which is most probably caused by the presence of different haplogroups. Based on the spatial distribution of haplogroups and the presence of shared haplotypes with other regions we assume that the Carpathian Basin is an admixture zone, which was repeatedly colonized from different sources in the past and present interglacials. We also found

an endemic haplogroup in Hungary. Shared haplotypes and pairwise  $\Phi_{ST}$  values support a strong genetic affinity between Italy and the Carpathian Basin and between Northern Europe (UK) and the Carpathian Basin.

- Although the genetic diversity is high in the Carpathian Basin, AMOVA showed low population substructure. This pattern parallels those seen in the cynipid gall inducers *Andricus quercustozae* and *A. quercuscalicis*.
- We tested for HAD using samples from the Mátra region (N = 55) of the Carpathian Basin only, considering the four most common cynipid hosts (*Andricus lucidus*, *A. lignicolus*, *A. infectorius*, *A. kollari*). AMOVA showed no sign of HAD, but further sampling is required to verify the generality of this result.
- Our data suggest a substantial genetic structure across the Western Palearctic, although it is important to emphasize that the present study is based on a single mitochondrial locus, and so inferences from the associated species tree must be made with caution. Regionally diagnostic lineages in each of Iberia (H7 and H3) and Iran (H8), and pairwise  $\Phi_{ST}$  values indicate strong divisions between Iberia and Central Europe, and between Iran and Turkey, which may be related to the mountain ranges of the Pyrenees and the Anatolian Diagonal. In contrast, we found a strong genetic affinity between Italy and the Carpathian basin, which suggests that the Alps do not prevent gall wasp dispersal. Our data support the colonization of Germany, France and the United Kingdom by westward expansion from Central Europe and not from Iberia, since haplogroups associated with Iberia (H3, H7) are not present in these northern postglacial populations. These patterns in many ways parallel those seen in other components of the oak gall wasp community, including gall inducers and their parasitoids.

## List of publications

### The present thesis is based on the following publications

- **Bihari P**, Sipos B, Melika G, Fehér B, Somogyi K, Stone GN and Péntzes Zs. 2011. Western Palearctic phylogeography of an inquiline gallwasp, *Synergus umbraculus*. *Biological Journal of the Linnean Society* **102**:750-764 IF: 2.04.
- Péntzes Zs, Melika G, Bozsóki Z, **Bihari P**, Mikó I, Tavakoli M, Pujade-Villar J, Fehér B, Fülöp D, Szabó K, Bozsó M, Sipos B, Somogyi K and Stone GN. 2009. Systematic re-appraisal of the gall-usurping wasp genus *Synophrus* Hartig, 1843 (Hymenoptera: Cynipidae: Synergini). *Systematic Entomology* **34**: 688-711 IF: 2.467.

### Other publications

- Ács Z, Challis RJ, **Bihari P**, Blaxter M, Hayward A, Melika G, Csóka Gy, Péntzes Zs, Pujade-Villar J, Nieves-Aldrey JL, Schönrogge K and Stone GN. 2010. Phylogeny and DNA barcoding of inquiline oak gallwasps (hymenoptera: Cynipidae) of the Western Palearctic. *Molecular Phylogenetics and Evolution* **55**: 210-225 IF: 3.556.
- Tavakoli M, Melika G, Sadeghi S, Péntzes Z, Assareh M, Atkinson R, Bechtold M, Mikó I, Zargaran M, Aligolizade D, Barimani H, **Bihari P**, Fülöp D, Somogyi K, Challis R, Preuss S, Nicholls J and Stone GN. 2008. New Species of Oak Gallwaps from Iran (Hymenoptera: Cynipidae: Cynipini). *Zootaxa* **1699**: 1-64 IF: 0.740.

- Álmos PZ, Horváth S, Czibula Á, Raskó I, Sipos B, **Bihari P**, Béres J, Juhász A, Janka Z and Kálmán J. 2008. H1 tau haplotype-related genomic variation at 17q21.3 as an Asian heritage of the European Gypsy population. *Heredity* **101**: 416–419. IF: 3.823.
- Melika G, Péntzes Zs, Mikó I, **Bihari P**, Ács Z, Somogyi K, Bozsóki Z, Szabó K, Bechtold M, Fári K, Fehér B, Fülöp D, Csóka Gy and Stone GN. A Kárpát-medence tölgyön élő gubacsdarazsai. Book chapter. A Kárpát-medence állatvilágának kialakulása - A Kárpát-medence állattani értékei és faunájának kialakulása, 2007, Budapest.