Tachinidae from central Italy:
an investigation of morphological and molecular diversity

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A deepening of our knowledge of biodiversity

has become more and more necessary in recent decades, especially in light of estimates that we have described just 20% of the living species on Earth (Mora *et al*. 2011, Wilson 2017). Fortunately, DNA barcoding (Hebert *et al*. 2003) has given a boost to the rate at which species can be recognized and delimited in hyperdiverse insects groups by detecting occurrences of both cryptic taxa (Hebert *et al*. 2004, Bickford *et al*. 2007) and highly variable taxa. Such studies have included the Tachinidae in recent years (Pohjoismäki *et al*. 2016, Lee *et al*. 2021).

During my Bachelor's studies at Sapienza University of Rome (Italy), I quickly became fascinated by the world of flies and especially the Tachinidae. Under the guidance of my supervisor, Prof. Pierfilippo Cerretti, I completed a B. Sc. thesis on tachinid systematics. I had by then become interested in ecobiology and began a Master's thesis on the topic of biodiversity assessment based on a total evidence approach, including morphological analysis and DNA barcoding. Prof. Cerretti was again my supervisor for this project. I recently completed my Master's and will briefly review its goals and discuss some of my findings.

My Master's thesis focused on the morphological and genetic characterization of inter- and intraspecific diversity of Tachinidae from the central Appennines in Italy. Two sites were chosen for the collection of specimens, both of them in protected areas bordering a national park, the Parco Nazionale D'Abruzzo, Lazio e Molise (PNALM), a significant hotspot for biodiversity in Italy. This park is just over 100kms east of Rome.

Figure 1. Malaise trap set in a small clearing among beech trees (1400m) in central Appennines, Italy.

Of course, such a project requires partnerships. Sampling was carried out with the help of Raggruppamento Carabinieri Biodiversità, the group responsible for protecting the natural resources in Italy and that manages some protected areas in Italian territory. Sequencing and molecular analyses were performed in collaboration with the Evolution Lab coordinated by Prof. Rudolf Meier of the National University of Singapore. Meier's research team has been a leader in DNA barcoding and molecular analyses on insects, and in particular Diptera, for many years (e.g., Meier *et al.* 2006, Srivathsan *et al*. 2018, Wang *et al*. 2018, Srivathsan *et al*. 2019, Yeo *et al*. 2020). Not only did they carry out the barcode sequencing for my project, but they also helped me with data processing, especially Sujatha Narayanan Kutty.

Fifteen Townes-style Malaise traps were set up in clearings among beech trees (Fig.1) at elevations from 1397 to 1637 metres above sea level. Flying insects were collected from July to October 2019 and the samples were preserved in bottles filled with 70% ethanol that I replaced every two weeks (Fig. 2).

Figure 2. Author at work during one trap check and bottle replacement.

In total, approximately 1000 tachinid flies were collected. From autumn 2019 to spring 2020 I dedicated myself to sorting and identifying the specimens to species level using the keys of Cerretti (2010) and with the assistance of the keys' author. After this was done, all the specimens were sent to Singapore University where the molecular analyses were carried out. DNA extraction and amplification followed new time-saving and inexpensive pipelines, including the use of QuickExtract™ DNA Extraction Solution that does not damage specimens, thus allowing for further morphological examination afterwards (Wang *et al*. 2018). The mitochondrial COI gene (313 bp, minibarcode) was sequenced using the cutting-edge MinION technology (Jain *et al*. 2016).

Sequences were compared with the ones stored in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and BOLD (http://www.boldsystems.org). I carried out barcode-based identifications considering matches with ≥97% identity as valid. In addition, we sorted sequences into putative species (mOTUs) by Objective Clustering (Meier *et al*. 2006) and then constructed a neighbor-joining tree to obtain a graphic representation of the clusters.

My thesis focused on the data concerning 303 of the roughly 1000 tachinid specimens collected during the study. The others are still being processed, having been delayed due to complications from the Covid-19 pandemic and the lockdown period that occurred in the spring-summer of 2020 in Italy. These data, although belonging to a subset of the collected specimens, are already providing some interesting observations resulting from the comparison of both morphological and molecular analyses. This small number of samples has also allowed me to become familiar with the techniques and with the different results that can be obtained from such a total evidence approach. I here briefly report some of the more interesting results that I achieved and wrote about in my thesis.

In 60% of the specimens, the morphological identification corresponds to the molecular one (i.e., there is a ≥97% match with one GenBank species). In the remaining 40% I found the following types of discrepancies between morphology and barcoding-based identifications:

1) some sequences correspond to species different from the morphological ones,
2) some sequences do not significantly match any barcode available in GenBank (i.e., no match ≥97%), and
3) others match multiple species (i.e., match ≥97% with more than one GenBank species, with the same percentage).

Figure 3. Specimen of *Lomachantha* Rondani with fore legs and part of abdomen orangish.

These problematic matches may have resulted from errors in the morphological identifications, or in the labelling of specimens during analyses, or even in the names associated with barcodes in GenBank or BOLD. However, some of the incongruences may, upon closer examination, reveal cryptic species or highly variable ones. For example, I identified three specimens as *Lomachantha parra* Rondani and one as *L. rufitarsis* Villeneuve (Fig. 3) based on morphology. The latter species is currently known only from the Middle East and Armenia. Even though the four flies differ in the colouration of their fore legs and abdomen (typically black in *L. parra* and orange in *L. rufitarsis*), they share the same barcode and were all identified (correctly we believe) as *L. parra* by comparison with sequences in GenBank. I found a similar situation with some specimens of *Phebellia* Robineau-Desvoidy. They differ in the presence/absence of well-developed setae on the posterodorsal surface of the hind coxae. According to the literature, the only species with these setae present is *P. nigricauda* Mesnil in Japan. Despite this morphological difference, the specimens all share the same barcode and are identified as *P. glauca* Meigen in GenBank (a species lacking setae on the hind coxae according to its description). Thus, my results likely indicate that in each of these two cases only one morphologically variable species is involved. I concluded at the end of all my analyses that the 303 specimens studied for my thesis belong to 73 species.

I also found that some congeneric species with similar and confusing characters are well divided by genetics (e.g., *Siphona* spp., *Dinera carinifrons* (Fallén) and *Dinera fuscata occidentalis* Ziegler), while others present a small genetic distance (i.e., <3%) even if they are clearly distinguishable by morphology (e.g., *Cylindromyia* spp.). In addition, the total evidence approach allowed me to also identify a possible new species, characterized by unique morphology and barcode (*Catharosia* sp.).

My early results agree with those obtained in recent works about Tachinidae in which morphological diversity is compared to differences in DNA barcodes (Pohjoismäki *et al*. 2016, Lee *et al*. 2021). However, there is still more to do: first of all, as soon as possible, my colleagues and I will resume work on the remaining 700 specimens and their barcodes, and then we will proceed with further analyses on morphology and sequences using also new investigative methods (e.g., other species delimitation methods and haplotype network analyses). By the end of the project we will be able to verify and compare the results of my thesis with those of the remaining specimens to better characterize this tachinid assemblage of the central Apennines. We can say at this point that the tachinid fauna of this study consists mostly of species that are recognizable by either morphology or barcodes but also has some cryptic species and highly variable species that can only be satisfactorily resolved by both morphology and barcodes.

This project is contributing new information about tachinid diversity in Italy and more barcodes to international databases. Moreover, it constitutes a starting point for further research into the hidden diversity and community composition of insects in our territory. In fact, once this work is completed, it would be interesting to extend the sampling on a national scale including different environments in order to get an overview of Italian insect diversity.

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REFERENCES

Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K. & Das, I. (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*, 22, 148–155.

Cerretti, P. (2010) *I tachinidi della fauna italiana (Diptera Tachinidae) con chiave interattiva dei generi ovest-paleartici*. Volumes I & II. Centro Nazionale Biodiversità Forestale, Verona. 573 pp. (Vol. I) + 339 pp. (Vol. II) + CD ROM.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & DeWaard, J.R. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, 270, 313–321.

Hebert, P.D.N., Penton, E.H., Burns, J.M., Janzen, D.H. & Hallwachs, W. (2004) Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of Americ*a, 101, 14812–14817.

Jain, M., Olsen, H.E., Paten, B. & Akeson, M. (2016) The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. *Genome Biology*, 17, Art. 239.

Lee, K.M., Zeegers, T., Mutanen, M. & Pohjoismäki, J. (2021) The thin red line between species – genomic differentiation of *Gymnosoma* Meigen, a taxonomically challenging genus of parasitoid flies (Diptera: Tachinidae). *Systematic Entomology*, 46, 96–110.

Meier, R., Shiyang, K., Vaidya, G. & Ng, P.K.L. (2006) DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. *Systematic Biology*, 55, 715–728.

Mora, C., Tittensor, D.P., Adl, S., Simpson, A.G.B. & Worm, B. (2011) How many species are there on Earth and in the ocean? *PLoS Biology*, 9 (8), e1001127.

Pohjoismäki, J.L.O., Kahanpää, J. & Mutanen, M. (2016) DNA barcodes for the northern European tachinid flies (Diptera: Tachinidae). *PLoS ONE*, 11 (11), e0164933.

Srivathsan, A., Baloğlu, B., Wang, W., Tan, W.X., Bertrand, D., Ng, A.H.Q., Boey, E.J.H., Koh, J.J.Y., Nagarajan, N. & Meier, R. (2018) A MinIONTM-based pipeline for fast and cost-effective DNA barcoding. *Molecular Ecology Resources*, 18, 1035–1049.

Srivathsan, A., Hartop, E., Puniamoorthy, J., Lee, W.T., Kutty, S.N., Kurina, O. & Meier, R. (2019) Rapid, large-scale species discovery in hyperdiverse taxa using 1D MinION sequencing. *BMC Biology*, 17, Art. 96.

Wang, W.Y., Srivathsan, A., Foo, M., Yamane, S.K. & Meier, R. (2018) Sorting specimen-rich invertebrate samples with cost-effective NGS barcodes: validating a reverse workflow for specimen processing. *Molecular Ecology Resources*, 18, 490–501.

Wilson, E.O. (2017) [Comment.] Biodiversity research requires more boots on the ground. *Nature Ecology & Evolution*, 1, 1590–1591.

Yeo, D., Srivathsan, A. & Meier, R. (2020) Longer is not always better: optimizing barcode length for large-scale species discovery and identification. *Systematic Biology*, 69, 999–1015.