



Large-scale DNA barcoding reveals cryptic diversity in eulophid wasps (Hymenoptera, Chalcidoidea, Eulophidae)

Emma Kärrnäs¹, Christer Hansson^{2,3}, Niklas Wahlberg^{1,2}

¹ Division of Biodiversity and Evolution, Department of Biology, Lund University, Kontaktvägen 10, Lund, Sweden

² Biological Museum (Entomology), Department of Biology, Lund University, Kontaktvägen 10, Lund, Sweden

³ The Natural History Museum, Life Sciences, Cromwell Road, London, United Kingdom

<https://zoobank.org/94C8DC86-5F78-4C68-A2C5-0071FCA922B2>

Corresponding author: Emma Kärrnäs (emma.karrnas@biol.lu.se)

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Abstract

Despite their ecological and economic importance as key regulators of insect populations worldwide, eulophid wasps remain one of the taxonomically most neglected groups of insects. As these minute parasitoid wasps exhibit an astonishing species diversity while being challenging to identify, relying on morphological characters alone has long been problematic. By using large-scale barcoding and molecular species delimitation analyses of 909 specimens, of which 641 were of European origin, we reveal a remarkable diversity of cryptic species in the two eulophid wasp genera *Oomyzus* Rondani and *Quadrastichus* Girault (Eulophidae, Tetrastichinae) present in Europe. We also present 40 new country records for 17 species and greatly improve the existing DNA barcode libraries of these two groups. Using the four different species delimitation methods ABGD, ASAP, GMYC and mPTP, the number of potentially undescribed European species detected in this study ranges from 20 to 34, of which 16 to 24 are cryptic. These results suggest that not even half of the European *Oomyzus* and *Quadrastichus* species are currently known, thus highlighting the need for further taxonomic work. However, the results also highlight issues with molecular species delimitations based on barcode data, such as incongruence across different analysis methods and potential barcode sharing between closely related species. Therefore, the results also challenge the validity of relying on a single molecular marker when detecting and subsequently describing new species, an increasing practise within modern taxonomy termed “turbo-taxonomy”.

Key words

ABGD, ASAP, cryptic species, GMYC, mPTP, *Oomyzus*, *Quadrastichus*, species delimitation

1. Introduction

Despite more than two centuries of taxonomic research, a majority of Earth’s extant biodiversity still remains to be discovered. While a staggering 1.7 million eukaryote species have been described to date (Ritchie 2022), it has been estimated that perhaps 9 million species exist, although the estimations of this number vary from 3 to 100 million (May 2010, Mora et al. 2011, Ritchie 2022). Out

of these millions of species, the most speciose group of all are the insects – according to current calculations, insects constitute about half of all known species diversity on Earth (IUCN 2023). Insects are thus keystone components in ecosystems throughout the world and have a major effect on their environment. With an ongoing sixth mass extinction and an alarming decline in insects (Dirzo

et al. 2014, Sánchez-Bayo and Wyckhuys 2019), there is an urgent need to establish and investigate Earth's insect diversity.

As a tool to accelerate this process, which includes delimiting, identifying and describing species, DNA barcoding was introduced two decades ago (Hebert et al. 2003b). Since its introduction this method has been frequently used to answer questions regarding evolution, ecology and conservation (Kress et al. 2015). One of its most frequent applications has been in the field of taxonomy, where it has been used for species delimitation and as an aid to discover cryptic species (Kress et al. 2015, DeSalle and Goldstein 2019). Some taxonomists even go as far as to base new species descriptions largely or solely on barcode data, a practise termed “turbo-taxonomy” (e.g. Butcher et al. 2012, Meierotto et al. 2019, Sharkey et al. 2021).

One insect group where species delimitation and the discovery of unknown species has been greatly facilitated by barcoding is the Hymenoptera (Johansson and Cederberg 2019, Hansson and Schmidt 2020, Sharkey et al. 2021, Praz et al. 2022, Sheikh et al. 2022). Although it is frequently claimed that Coleoptera is the most species-rich insect order, estimations suggest that Hymenoptera is even more speciose, largely due to the vast number of undescribed species of parasitoid wasps (Forbes et al. 2018). One of the most understudied groups of parasitoid wasps is the hyperdiverse superfamily Chalcidoidea, suggested to encompass perhaps as many as 500 000 species, of which 27 000 have been described to date (Heraty 2009, UCD Community 2023).

Since a recent revision of the Chalcidoidea (Burks et al. 2022), Eulophidae is now considered the most speciose family of chalcidoid wasps. With its >6500 extant species it encompasses almost a quarter of the known diversity of the superfamily (UCD Community 2023). Eulophids are found in virtually all terrestrial habitats on every continent except Antarctica (Rasplus et al. 2020). Recorded information suggests that the majority of eulophid species develop as parasitoids on various groups of insects and arachnids. A large number of the species in Eulophidae belong to the subfamily Tetrastichinae, that has a host spectrum of 100 insect families in ten insect orders (LaSalle 1994). Some of the hosts are considered pests in agriculture and forestry, causing major damage to various economically valuable crops (UCD Community 2023). Despite their ecological and economical importance, tetrastichines have been taxonomically neglected, mainly because of the large number of morphologically similar species (Hansson and Schmidt 2020). However, the use of DNA barcoding shows promising potential in species delimitation and detection, showcased by the study by Hansson and Schmidt (2020) of the genus *Tetrastichus* Haliday (Eulophidae, Tetrastichinae). This genus includes several morphologically similar and hard-to-distinguish species, where morphological data combined with barcode data revealed 50 undescribed species in Europe, more than doubling the number of recorded species in the region. The study hinted at the immense number of eulophid species still awaiting discovery, even in relatively well-studied regions such as Europe.

Given that, to our knowledge, no tetrastichine genus apart from *Tetrastichus* has been studied in detail using molecular data, we wanted to examine whether the hidden diversity in *Tetrastichus* represents a general pattern. To investigate this, we selected the two tetrastichine genera *Oomyzus* Rondani and *Quadrastichus* Girault (Fig. 1B, C), for large-scale barcoding and species delimitation analysis. Both genera are closely related to *Tetrastichus* (Rasplus et al. 2020). *Oomyzus* is a small genus with only eleven species recorded from Europe, and *Quadrastichus* includes 22 European species (Graham 1991). Species of both genera develop as solitary or gregarious endoparasitoids in their respective host: *Oomyzus* mainly targets different families of Coleoptera, e.g. weevils (Curculionidae) and ladybirds (Coccinellidae), while *Quadrastichus* targets different species of gall midges (Cecidomyiidae), with one species recorded from mites (Acari) in galls (Graham 1991). Based on DNA barcode data from 641 specimens from 17 European countries (Table S1) we present the first hypothesis regarding the number of species of *Oomyzus* and *Quadrastichus* present in Europe.

2. Material and Methods

2.1. Sampling

A total of 359 specimens of *Oomyzus* and *Quadrastichus* (preserved in 95% ethanol) were sampled for this study. The specimens were caught either by sweeping vegetation according to Noyes (1982) or with Malaise traps (Townes 1972). Some specimens were reared from galls of cecidomyiid hosts. The material is mainly from various localities in Sweden, but also from localities in Romania, France, Norway and United Kingdom (Fig. 1A), collected between 2003 and 2023. Detailed information about all specimens may be found in Table S1. A substantial proportion of the material was collected during the Sweden Insect Inventory Program (SIIP) 2018–2019, a follow-up inventory of the Swedish Malaise Trap Project (SMTP) 2003–2006 (Karlsson et al. 2020). Voucher specimens are stored at the Biological Museum, Lund University (MZLU), the Natural History Museum, London (NHMUK) and at Station Linné, Öland, see Table S1. Specimens were identified to species with keys in Graham (1991). When reporting new country records by matching unidentified public barcode sequences to a known species, only sequences that were >99% similar to another sequence verified by morphology were included.

2.2. DNA extractions, PCR and sequencing

Genomic DNA was extracted non-destructively from the specimens by placing the entire specimen in overnight lysis in a ThermoShaker set at 300 rpm, using the Nu-

cleospin Tissue kit (Machery-Nagel, Düren, Germany). After lysis, the specimen was put in absolute ethanol and treated with hexamethyldisilazane (HMDS) and then dry-mounted, according to Heraty and Hawks (1998) and Noyes (1982). The extraction was done according to the manufacturer's protocol, with the following modifications: (1) elution buffer was preheated to 56°C and (2) elution was done in two steps of 50 µl and incubated for 15 min at 56°C.

Amplification of the barcode region of *COI* (*COI-5P*) was carried out with any of the two universal primer pairs HybLCO/HybHCO (Wahlberg and Wheat 2008) or Lep-F1/Lep-R1 (Hebert 2004). The thermocycling profile of the PCR was the following: initial denaturation of 5 min at 95°C, 40 cycles of 94°C for 30 s, 50°C for 30 s

and 72°C for 90 s, followed by a final extension period of 72°C for 10 min. Before being sent for sequencing at Macrogen Europe (Amsterdam, Netherlands), plates were cleaned using ExoSAP-IT Express (Thermo Fisher Scientific) by adding 1 µl of the master mix to 10 µl of PCR product, which was then incubated at 37°C for 45 min, followed by 85°C for 15 min. The plates were sent for Sanger sequencing at either the facility of Macrogen Europe or Microsynth AG (Balgach, Switzerland).

The resulting sequences were inspected and manually edited using Geneious (2023.2.1) and deposited in The Barcode of Life Data System (BOLD: EULOP001-24–EULOP361-24 (DS-QUADOOM)) and GenBank (PV356342–PV356700).

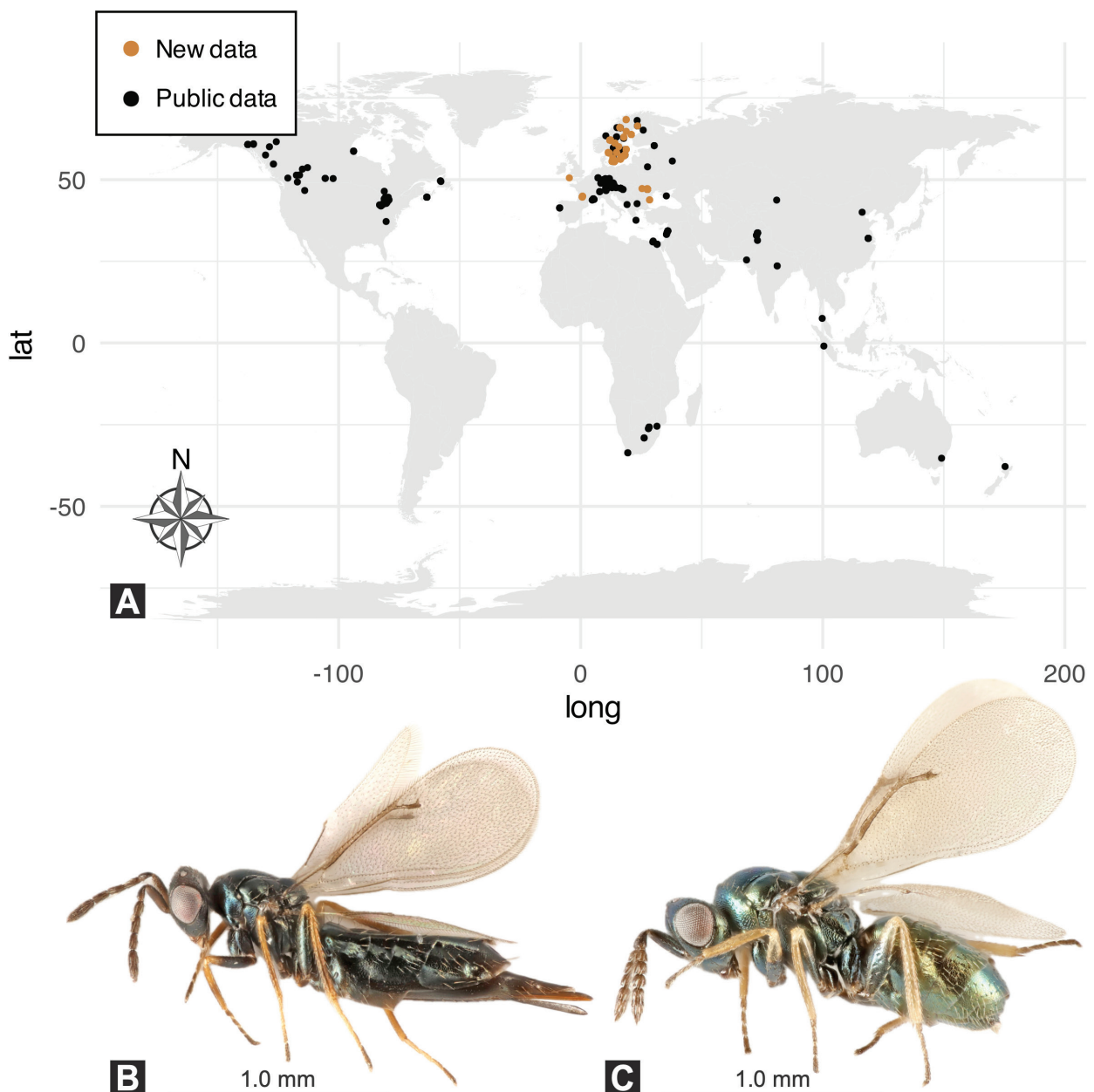


Figure 1. A Map showing the geographic distribution of newly generated barcodes of *Oomyzus* and *Quadrastichus* (brown), and already publicly available barcodes in BOLD and/or GenBank (black). The map was done with the R package maps v3.4.2.1 (Becker et al. 2024). B The focal genus *Quadrastichus* (*Q. vacuna* (Walker)). C The focal genus *Oomyzus* (*O. incertus* (Ratzeburg)).

2.3. Alignment and phylogenetic inference

To obtain a more comprehensive review of the relationships within and between *Oomyzus* and *Quadrastichus*, the databases BOLD (<https://boldsystems.org/>) and GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) were searched for all public records of *COI-5P* of the two genera. These sequences were manually inspected, translated and checked for stop codons, and compared to sequences verified by morphology for apparent identification errors. In a preliminary inferred phylogenetic tree including various outgroups, public sequences that were closely clustering with eulophid genera other than *Oomyzus* or *Quadrastichus* were deemed as identification errors and were subsequently removed, resulting in a total of 550 sequences being added to the final dataset. As we did not have access to most specimens of the public sequences, we could not examine these morphologically and the specimens' identity was thus recorded as given in the database.

The combined dataset was aligned with MAFFT v7.520 (Kato and Standley 2013) and manually inspected for alignment errors. Sequences with >60% missing data were disregarded. The maximum likelihood (ML) phylogenetic inference was done in IQ-TREE2 v2.2.0 with 1000 UFB and standard model selection with ModelFinder followed by tree inference (Hoang et al. 2017, Kalyanamoorthy 2017, Minh et al. 2020). The model K3Pu+F+I+G4 was chosen according to the BIC score. The species *Hyssopus geniculatus* (Hartig) (Eulophidae, Eulophinae) was chosen as outgroup for the phylogeny. The resulting phylogenetic tree was visualised and edited in FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>) and Inkscape v1.1 (<https://inkscape.org/>).

2.4. Species delimitation analysis

We used four species delimitation methods to evaluate the number of putative species, or molecular operational taxonomic units (MOTUs), in the dataset: the two cluster-based methods Automated Barcode Gap Discovery (ABGD) (Puillandre et al. 2012) and Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2021); the tree-based methods General Mixed Yule Coalescent model (GMYC) (Pons et al. 2006) and multi-rate Poisson Tree Processes (mPTP) (Kapli et al. 2017). These methods do not require any prior information on species identities and are appropriate for a single molecular marker, such as the animal barcode region *COI-5P*. They were chosen based on their popularity and strong performances in other studies (Talavera et al. 2013, Kapli et al. 2017, Magoga et al. 2021, Ranasinghe et al. 2022).

The species delimitation analyses with ABGD and ASAP were performed in December 2024 on their respective web server (ABGD: <https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>; ASAP: <https://bioinfo.mnhn.fr/abi/public/asap>) using the JC69 model and default set-

tings. Both methods use pairwise genetic distances for detecting a barcode gap between species, i.e. an interspecific sequence divergence larger than the intraspecific sequence divergence. A distance threshold is then estimated from this gap, which is used to recursively partition the sequences into putative species. The two methods use different algorithms, where ABGD, in contrast to ASAP, requires the maximal genetic intraspecific divergence a priori for detecting the barcode gap. While the results of ABGD are unranked, ASAP provides a score for every partition (Puillandre et al. 2021).

Instead of relying on thresholds, the two tree-based species delimitation methods GMYC and mPTP take the phylogeny into consideration when delimiting species (Pons et al. 2006, Kapli et al. 2017). For both methods, the tree generated by IQ-TREE was used as input. For species delimitation with GMYC, the tree was first transformed into an ultrametric tree with the phylogenetic penalized likelihood program TreePL (Smith and O'Meara 2012), with the age of the most recent common ancestor of the outgroup and the remaining taxa set between 96 million years (minimum age) and 119 million years (maximum age), as obtained from Cruaud et al. (2024). The subsequent species delimitation with GMYC was performed in R v4.4.2 (R Core Team 2024) with the R package “splits” v1.0–20 (Ezard et al. 2021), using a single-threshold model and an interval of 0–10.

For species delimitation with mPTP, a ML program allowing for the intraspecific divergence to vary among species (Kapli et al. 2017), the minimum branch length was first calculated from the alignment of sequence data. The suggested minimum branch length (–minbr 0.0023746901) was then used as input for the subsequent analysis, which was run with the multi-rate option, assessing the support of the different MOTUs with 10 independent Markov chain Monte Carlo (MCMC) runs, each running for 100 000 000 iterations, sampling every 5000 iterations and discarding the first 20 000 iterations as burn-in.

For the different species delimitation methods, the mean pairwise intra- and interspecific genetic distances were calculated with the R package “BarcodingR” (Zhang et al. 2016), using the function “barcoding.gap” and the K2P model for genetic distance (“K80”).

3. Results

The final dataset consisted of 909 barcode sequences from 29 countries, of which 845 were unique haplotypes. The majority, 641 of 909 (71%), were of European origin, including all newly generated barcodes (Fig. 1A). During the scope of this study, we sampled 22 of the 34 described European species in the genera *Oomyzus* and *Quadrastichus*: 9 out of 12 European *Oomyzus* species and 13 out of 22 European *Quadrastichus* species. In contrast, we recovered 40–56 MOTUs with a European distribution (Fig. 2A).

The different species delimitation methods resulted in a different number of MOTUs, with the total number of putative species ranging from 40 to 59. ABGD recovered 40 MOTUs ($P = 0.021544$), ASAP 51, GMYC 59 and mPTP 54 MOTUs. Out of these, 22 MOTUs were obtained with all methods (Fig. 3). The number of singletons was consistent, varying from 12 (ABGD, mPTP), to 14 (ASAP) and 15 (GMYC), which corresponds to 22–30% of the total number of MOTUs (Fig. S1). The mean pairwise intra- and interspecific genetic distances were 3.9% and 7.6% for ABGD, 3.7% and 7.8% for ASAP, and 4.9% and 9.4% for both GMYC and mPTP, respectively.

Of the 40–56 delimited European MOTUs, 50–61% represent species potentially new to science, with the number ranging from 20 as delimited with ABGD, 30 with ASAP, 31 with mPTP and to 34 with GMYC (Fig. 2A). The number of possible undescribed species was estimated to be between 10 (ABGD) and 20 (GMYC) in *Quadrastichus*, and either 10 (ABGD) or 14 (ASAP, GMYC, mPTP) in *Oomyzus* (Fig. 2B). When also considering the non-European MOTUs, an additional 0–3 possibly undescribed species were detected in the dataset (Fig. 3). A majority, 16–24, of the 20–34 possibly undescribed species are cryptic, i.e. several MOTUs were identified as the same morphological species given the current species definitions. A few nominal species harbour the bulk of the possible cryptic species diversity with *Q. anysis* (Walker) forming 2–3 MOTUs, *Q. praecox* (Graham) forming 3 MOTUs, *Q. vacuna* (Walker) forming 3–8 MOTUs, and *O. incertus* (Ratzeburg) forming 8–10 MOTUs across the different species delimitation methods (Fig. 3).

Interestingly, all methods returned a MOTU containing both *O. galerucivorus* (Hedqvist) and *O. repentinus* (Graham), two morphologically distinct species, with the tree

showing *O. repentinus* nesting within *O. galerucivorus* (Figs 3, S1). The *O. repentinus* clade had a 0.9% genetic distance to its nearest neighbour. Furthermore, all methods apart from GMYC lumped two distinct morphological species into single MOTUs: mPTP lumped *Quadrastichus* sp. 4 + *O. propodealis* Graham into a joint MOTU, ASAP placed *O. spiraculus* Song, Fei & Cao + *O. scaposus* (Thomson) in a single MOTU and ABGD lumped *O. spiraculus* + *O. scaposus*, *Oomyzus* sp. + *O. propodealis*, *Quadrastichus* sp. 1 + *Q. pedicellaris* (Thomson) and *Quadrastichus* sp. 2 + *Q. praecox* into single MOTUs. In total, ABGD delimited four non-monophyletic MOTUs, while ASAP delimited one non-monophyletic MOTU, according to the inferred phylogenetic tree of the two groups (Fig. 3).

The phylogenetic tree as inferred by *COI-5P* provides the first preliminary hypothesis for the relationships within *Oomyzus* and *Quadrastichus*. While the bootstrap support overall is moderate, many of the delimited MOTUs do have high support (UFB >95) (Hoang et al. 2017) (Fig. 3).

Finally, the morphological identifications and barcoding performed within the scope of this study has resulted in 18 species having their names added to a public barcode database. This includes the outgroup *Hyssopus geniculatus*, 6 out of 9 *Oomyzus* species and 11 out of 13 *Quadrastichus* species. Furthermore, the morphological identifications and subsequent analysis of the barcode sequence data confirms 40 new country records for 17 species of *Oomyzus* and *Quadrastichus*, spanning 18 countries (Table S2). Of these new country records, 16 were corroborated by morphology and 15 were previously unidentified sequences (i.e. unidentified to species level) matched to a species name for the first time (Table S2).

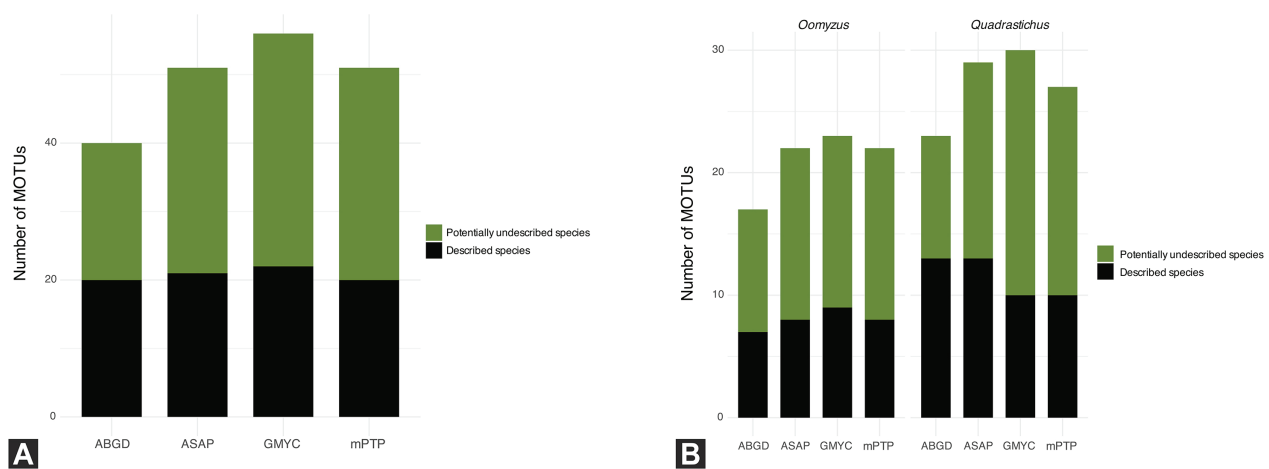


Figure 2. **A** The number of MOTUs delimited with the four different species delimitation methods ABGD ($P = 0.021544$), ASAP, GMYC and mPTP, excluding the outgroup. Only MOTUs with a European distribution are shown. MOTUs including already described species are shown in black, and MOTUs representing potentially undescribed species are shown in green. The number of potentially undescribed species was calculated by counting the MOTUs representing cryptic species (i.e. multiple MOTUs morphologically identified as a single species), and those exclusively containing taxa unidentified to any currently described species. **B** The number of MOTUs delimited for the genera *Oomyzus* and *Quadrastichus*, using the four different species delimitation methods ABGD ($P = 0.021544$), ASAP, GMYC and mPTP, excluding the outgroup.

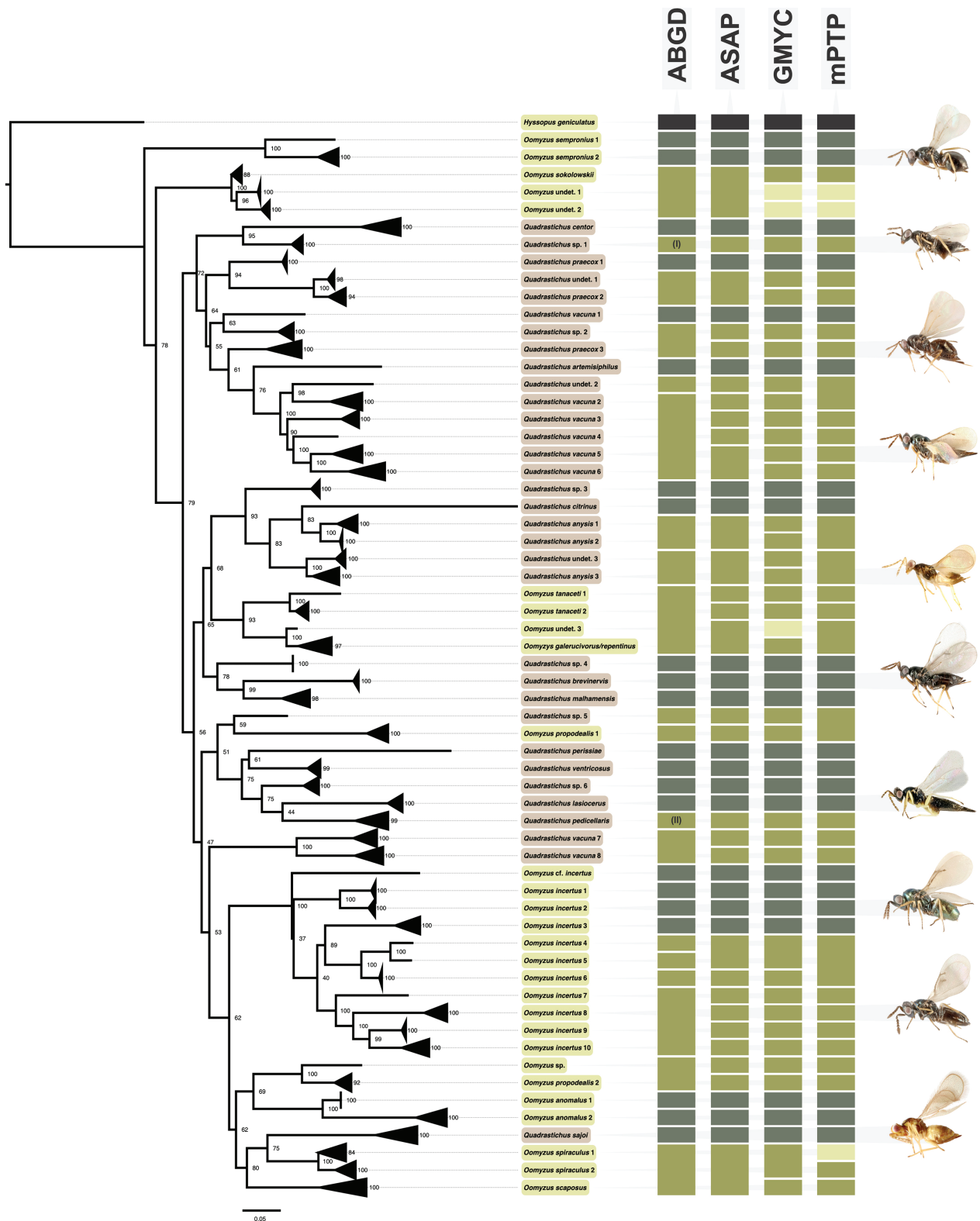


Figure 3. Maximum likelihood tree with MOTUs as delimited by four different methods: ABGD, ASAP, GMYC and mPTP. Bootstrap support is shown on the branches; the scale bar shows genetic distance. Identical MOTUs delimited by all methods are coloured in dark grey while MOTUs with incongruences across methods are coloured in green; the outgroup is coloured in black and MOTUs found exclusively outside of Europe are coloured in pale green. One MOTU recovered by ABGD is highly polyphyletic with respect to the inferred tree and is thus found in two parts, indicated by (I) and (II). For a tree showing all delimited taxa, see Figure S1.

4. Discussion

The results of this study show how little we know about the true diversity of eulophid wasps, even in a relatively well-studied region such as Europe. By barcoding hundreds of *Oomyzus* and *Quadrastichus*, this study takes a first important step towards a better understanding and a much-needed systematic review of these taxonomically neglected groups. The results suggest that there is a high number of undescribed *Oomyzus* and *Quadrastichus* species, as 20–34 of the delimited MOTUs may correspond to unknown species (Fig. 2A). Considering that the geographical sampling was somewhat limited, and that roughly a third of the European species were not sampled in this study, the true number of species present in Europe is most certainly much higher than the current checklists indicate (Graham 1991). This is in line with other barcode studies on parasitoid wasps, which frequently find a large number of cryptic and unknown species when using molecular markers for the first time (e.g. Hansson and Schmidt 2020, Sheikh et al. 2022).

Precisely which MOTUs represent valid species is not evident without further taxonomic work, which we are currently undertaking. The 22 MOTUs which were congruent across different delimitation methods likely represent valid species, as most are also supported by distinguishing morphological characters. However, the different methods delimited a variable number and composition of species (Figs 2, 3), which highlights one of the main issues with a turbo-taxonomic approach to describing species based on a single molecular marker – in particular when delimiting species with a single molecular species delimitation method (see Meier et al. 2022). Many other studies show similar incongruences across methods, and there seems to be no consensus as to which method is the most accurate – all methods may result in both over-splitting and lumping of morphological species (Kekkonen et al. 2015, Galimberti et al. 2021, Magoga et al. 2021, Ranasinghe et al. 2022, Blair and Bryson 2017). Nevertheless, in this study ABGD yielded more unlikely MOTUs than any of the other methods by lumping several pairs of morphologically distinct groups into single MOTUs, and by also delimiting various non-monophyletic MOTUs. In contrast, GMYC performed the least amount of lumping of morphologically distinct groups and was subsequently the method that yielded the highest number of species in this study. However, GMYC has been shown to have a tendency to delimit an unrealistic high number of species in other studies (Paz and Crawford 2012, Talavera et al. 2013, Kekkonen et al. 2015, Song et al. 2018). ASAP and mPTP both appeared to perform relatively well in terms of lumping few morphological species, and the two methods often generated identical MOTUs (Fig. 3). Both methods have been shown to perform well in a number of studies (e.g. Kapli et al. 2017, Magoga et al. 2021, Puillandre et al. 2021, Ranasinghe et al. 2022), and mPTP has been shown to be preferable for highly uneven datasets such as the one in this study (Blair and Bryson 2017). In

addition, mPTP allows for the intraspecific divergence to vary among species (Kapli et al. 2017), which is expected as species have different population sizes and divergence times (Yang and Rannala 2017).

Strikingly, all four species delimitation methods showed a high mean pairwise intraspecific divergence, varying between 3.7 and 4.9% depending on the species delimitation method. This appears somewhat unlikely as the intraspecific barcode divergence in animals commonly is reported to be much lower, <1%, with an interspecific divergence often >2% (Avice 2000, Hebert et al. 2003a, Hausmann et al. 2013). However, it has been shown that insects, and in particular hymenopterans, commonly have a large intraspecific genetic variation, with almost a third of all species exhibiting >3% genetic variation (Zhang and Bu 2022). In turn, the mean interspecific pairwise divergence to congeneric species is also high, at 11.5% (Hebert et al. 2003a). A recent study on the parasitoid wasp *Lariophagus distinguendus* (Förster) (Chalcidoidea, Pteromalidae) showed that reproductive isolation (RI) was virtually absent between strains with up to 2.8% sequence divergence of *COI*, while being strong to complete between strains by at least 7.2% *COI* divergence (Pollmann et al. 2023). Moreover, in *Gonatocerus morrilli* Howard (Chalcidoidea, Mymaridae), RI was complete at 5.4–6.5% pairwise divergence (de León et al. 2006).

Interestingly, all delimitation methods failed to delimit the two closely related, but morphologically distinct, species *O. galerucivorus* and *O. repentinus*. This is not surprising given that the *O. repentinus* clade nests within *O. galerucivorus* in the barcode tree, with a genetic distance of <1% to the nearest *O. galerucivorus* neighbour, further pointing towards the shortcomings of using a single marker such as *COI* for species delimitation. Although many studies confirm the usefulness of distinguishing species with DNA barcode data, many studies also demonstrate cases where it falls short due to incomplete lineage sorting or hybridisation, known as barcode sharing (Hebert et al. 2003a, Meyer and Paulay 2005, Hausmann et al. 2013, Talavera et al. 2013, Galimberti et al. 2021). Therefore, many studies highlight the importance of using a multi-locus dataset for a more correct species delimitation (e.g. Klimov et al. 2019, Blair and Bryson 2017, Liu et al. 2017), which we also propose as a future approach for this group.

Beyond suggesting new species hypotheses for *Oomyzus* and *Quadrastichus*, the results of this study also provide a much-improved public barcode library (Table S1), an improved understanding of the species distributions (Table S2) and a first hypothesis of the phylogenetic relationships within and between *Oomyzus* and *Quadrastichus* (Fig. 3). The latter suggest that neither genus is monophyletic. However, as the phylogenetic tree is inferred using a single marker with many clades only having moderate support, these results should be interpreted with caution. For resolving the evolutionary relationships, a large-scale phylogeny both including more genes and more outgroups would be necessary, something we currently are working on.

5. Conclusions

This study greatly improves our poor knowledge of European *Oomyzus* and *Quadrastichus* by providing new barcode and species distribution data and by proposing a first phylogenetic hypothesis of the two genera. It also shows the likely presence of numerous undescribed species, including many cryptic ones, which forms an avenue for ongoing taxonomic work on these groups. Although species delimitation analyses based on barcode data are useful for exploring virtually unknown groups of organisms by speeding up the identification of undescribed species, this study underlines the shortcomings of relying on a single molecular marker and in particular relying on a single molecular species delimitation method when describing new species. We therefore recommend relying on multiple loci instead of a single marker, and when describing species, taking an integrative taxonomic approach, combining molecular data with morphological, biological, ecological and geographical distribution data.

6. Declarations

Author contributions. EK: Conceptualisation, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing – Original draft, Writing – Review and Editing, Visualisation, Project administration, Funding Acquisition. CH: Conceptualisation, Validation, Resources, Writing – Review and Editing, Supervision, Funding Acquisition. NW: Conceptualisation, Methodology, Writing – Review and Editing, Supervision, Project administration, Funding Acquisition.

Data availability. All data generated by or analysed in this study are available in BOLD and/or GenBank.

Competing interests. The authors declare no competing interests.

Permissions. The authors had all necessary permissions.

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Supplementary Material 1

Figure S1

Authors: Kärrnäs E, Hansson C, Wahlberg N (2025)

Data type: .pdf

Explanation notes: Maximum likelihood tree of all delimited taxa with MOTUs as delimited by four different methods: ABGD, ASAP, GMYC and mPTP. Bootstrap support is shown on the branches, excluding branches within delimited species; the scale bar shows genetic distance. Newly generated barcodes are coloured in black and barcodes that were sourced from BOLD or GenBank are coloured in grey. Identical MOTUs delimited by all methods are coloured in dark grey while MOTUs with incongruences across methods are coloured in green; the outgroup is coloured in black and MOTUs found exclusively outside of Europe are coloured in pale green. One MOTU recovered by ABGD is highly polyphyletic with respect to the inferred tree and is thus found in two parts, indicated by (I) and (II).

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Supplementary Material 2

Tables S1, S2

Authors: Kärrnäs E, Hansson C, Wahlberg N (2025)

Data type: .zip

Explanation notes: **Table S1.** Data for all specimens included in the study, including BOLD/GenBank accession codes, morphological and molecular identification data, collection data and museum deposition data. — **Table S2.** New country records for *Oomyzus* and *Quadrastichus*. The table includes BOLD/GenBank accession codes and collection and identification data.

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