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ABSTRACT

Eulophidae is a hyper-diverse family of chalcidoid wasps with 324 genera, about 5300 described species and probably thousands of others to be described. Until now, the absence of unequivocal morphological apomorphies and the low resolution provided by the handful of Sanger sequenced genes have hampered the reconstruction of phylogenetic relationships within the family. Here, we used ultra-conserved elements and their flanking regions to resolve relationships among 84 species of eulophids included in 63 genera representing all subfamilies and most tribes, plus 15 outgroups. Our analyses recover all traditional Eulophidae subfamilies and tribes with high support and globally agree with the traditional classification of the family. Our results confirm that Eulophinae + Tetrastichinae is the sister group of (Opheliminae + Entiinae) + Entedoninae. At the generic level, our analyses provide high support for intergeneric relationships for which morphology and Sanger markers previously failed to provide resolution. Our results also confirm that *Trisecodes* does not group with Eulophidae and may not belong to this family; however, its correct classification still awaits a large-scale phylogenomic hypothesis for Chalcidoidea. This work opens new avenues towards a better understanding of the evolutionary history, biogeography and evolution of host–parasitoid associations in this hyper-diverse family of chalcidoid wasps.

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Supplemental material for this article can be accessed here.

Introduction

With the exception of Pteromalidae, no other family of Chalcidoidea has evolved such a diversity of species and range of biological and ecological disparity as the Eulophidae. With 324 genera, about 5300 described species and probably thousands of others to be described (Noyes 2018), eulophids represent one of the most diverse family of chalcidoid wasps. The family is represented in every region outside the Antarctic and in nearly all types of terrestrial habitats. In addition, eulophids exhibit a wide spectrum of life-history characteristics. They can be gall-makers (LaSalle 2005; Kim and LaSalle 2008; Rasplus et al. 2011) or phytophagous on leaves, twigs or seeds although they are mostly parasitoids of larval and nymphal stages or adults of insects. Numerous species are primary parasitoids of leaf-feeding lepidopteran larvae as either idiobiont ectoparasitoids or koinobiont endoparasitoids, but many species are known to be ectoparasitic hyperparasitoids. According to current knowledge, species are often monophagous, but species groups or genera can be generalists or specialised on specific hosts (e.g. on Thysanoptera, Aleyrodidae). Multiple species belonging to different species groups are oophagous on Coleoptera, Hemiptera or Dictyoptera. Tetrastichinae develops at the expense of no less than 100 families of hosts belonging to 10 orders of insects (LaSalle 1994). Eulophidae mostly parasitise endophytic larvae of insects (Diptera, Coleoptera, Lepidoptera, Hymenoptera) but can also attack eggs of spiders, gall-forming mites or nematodes (Berg et al. 1990). Therefore, eulophids are essential for regulating populations of phytophagous insects in natural ecosystems, and a number of eulophid species have been used in biological control programmes to regulate pests (Hoy and Nguyen 2000; Jaramillo et al. 2005; Duan et al. 2013).

The monophyly of Eulophidae has never been challenged. However, only few synapomorphies support this traditional and morphological view (Burks et al. 2011). Furthermore, most diagnostic characters that support the monophyly of the family are reductions such as the number of tarsomeres or antennomeres or are homoplastic across Chalcidoidea [e.g. that the mesotrochantinal plate is inflected and separated from the metasternum by a membrane, which also occurs in Agaonidae (Heraty et al. 2013)]. Eulophidae is traditionally classified into five subfamilies (Entedoninae, Entiinae, Eulophinae, Opheliminae and Tetrastichinae) (Bouček 1988). Recently, Doğanlar and Doğanlar (2013) added a sixth subfamily (Ceranisinae) for a few genera with reduced mandibles that develop as larval parasitoids of Thysanoptera. However, this subfamily was subsequently synonymised with Entedoninae by Triapitsyn (2015). All subfamilies of Eulophidae are also rather poorly defined morphologically. Indeed, most morphological characters used to define suprageneric entities within Eulophidae appear to be highly homoplastic and variable at different taxonomic levels. Therefore, several subfamilies (e.g. Tetrastichinae, Entedoninae) and tribes (e.g. Entedonini) cannot be defined by a single or a set of morphological synapomorphies. Nevertheless, most molecular studies using Sanger sequencing have recovered Eulophidae as well as most subfamilies as monophyletic. However, inter-tribal and inter-generic relationships are still poorly resolved, often due to poor taxon sampling (Campbell et al. 2000; Gauthier et al. 2000; Munro et al. 2011; Heraty et al. 2013).

Several studies have examined the generic relationships within Eulophidae (Schauff 1991; Gauthier et al. 2000; Gumovsky 2002, 2011; Sha et al. 2006). A recent study that

combined molecules and morphology significantly enhanced our understanding of the evolutionary history of the family (Burks et al. 2011). In this work (54 species included), as well as in Munro et al. (2011) (27 species included), the relationships between the subfamilies were well resolved, and the authors proposed that Eulophinae + Tetrastichinae was the sister group of (Opheliminae + Entiinae) + Entedoninae. Relationships within subfamilies were less well resolved. Until now, the absence of unequivocal morphological apomorphies and the low-resolution power of the sequenced genes have hampered the reconstruction of phylogenetic relationships within eulophid subfamilies. The challenge of elucidating evolutionary relationships among genera is further complicated by the homoplastic nature of multiple characters used to define the generic limits. Therefore, recent molecular advances combined with novel computational approaches represent an interesting opportunity to better understand the tree of life of Eulophidae. Indeed, it is now possible to sequence hundreds of markers at a reasonable cost to reduce stochastic errors and better resolve phylogenetic relationships.

Many methods are available to gather genome-scale data among which sequence capture is a scalable and affordable method for broad-scale phylogenetics (Mamanova et al. 2010; Lemmon and Lemmon 2013). Among the main genomic regions that can be targeted, ultra-conserved elements (UCEs, Faircloth et al. 2012; McCormack et al. 2012) have been extensively used to reconstruct phylogeny in multiple groups of Hymenoptera, including Chalcidoidea (Blaimer et al. 2015, 2016a; Jesovnik et al. 2017; Branstetter et al. 2017a, 2017b; Bossert and Danforth 2018; Bossert et al. 2019; Cruaud et al. 2019, 2020). UCEs are highly conserved regions of genomes shared among distant taxa, and specific set of probes have been designed to capture these regions in Hymenoptera (Faircloth et al. 2015). The phylogenetic utility of UCEs and their more variable flanking regions has been demonstrated on multiple evolutionary scales, and they may also prove useful in contributing to decipher generic relationships within Eulophidae. Interestingly, the capture of UCEs does not necessarily require fresh specimens or high-quality DNA. Museum samples have been indeed successfully used (McCormack et al. 2016; Blaimer et al. 2016b). Finally, fast and efficient protocols have been optimised to capture UCEs on individual tiny insects without destruction of the specimens, in a few days for a cost of about 50 USD per individual (Cruaud et al. 2019).

Until now, molecular studies of the family were based on data sets composed of a few tens of morphological characters, and sequences of about three to four genes obtained with traditional Sanger sequencing on less than 60 species. In this paper, and for the first time, we used hundreds of pangenomic markers (UCEs) and numerous taxa to investigate the Eulophidae tree of life with a data set representative of all subfamilies and most tribes. This work is a first step towards a better understanding of the evolutionary history of this hyper-diverse family.

Materials and methods

Sampling

Our sampling comprises 84 species included in 63 genera representing all subfamilies and most tribes plus 15 outgroups (Table S1). Samples were collected by authors of this paper

or borrowed from the Queensland Museum (Australia) and the Australian National Insect Collection, Canberra. Three rare tribes of Eulophidae could not be included in our data set, namely Platytetracampini (Entedoninae),and Gyrolasomyiini (Tetrastichinae) each represented by one extant Australasian genus.

DNA extraction, library preparation and sequencing

DNA was extracted non-destructively and vouchers were subsequently remounted on cards. DNA was extracted using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's protocol with a few modifications detailed in Cruaud et al. (2019). Vouchers were deposited as detailed in Table S1. Library preparation followed Cruaud et al. (2019). Briefly, input DNA was sheared to a size of ca 400 bp using the Bioruptor® Pico (Diagenode). End repair, 3′-end adenylation, adapter ligation and PCR enrichment were then performed with the NEBNext Ultra II DNA Library prep kit for Illumina (NEB). Adapters that contained amplification and Illumina sequencing primer sites, as well as a nucleotide barcode of 5 or 6 bp long for sample identification, were used to tag samples. Pools of 16 samples were made at equimolar ratio. Each pool was enriched using the 2749 probes (v1) designed by Faircloth et al. (2015) using a MYbaits kit (Arbour Biosciences) and following the manufacturer's protocol. The hybridisation reaction was run for 24 h at 65°C. Post-enrichment amplification was performed on beads with the KAPA Hifi HotStart ReadyMix. The enriched libraries were quantified with Qubit, an Agilent Bioanalizer and qPCR with the Library Quantification Kit – Illumina/Universal from KAPA (KK4824). They were then pooled at equimolar ratio. Paired-end sequencing (2*300 bp) was performed on an Illumina Miseq platform at UMR AGAP (Montpellier, France).

Data analysis

The analytical workflow followed Cruaud et al. (2019). Quality control checks were performed with FastQC v.0.11.2 (Andrews 2010). Quality filtering and adapter trimming were performed with Trimmomatic-0.36 (Bolger et al. 2014). Overlapping reads were merged using FLASH-1.2.11 (Magoc and Salzberg 2011) and demultiplexing was performed with a bash custom script (no mismatch in barcode sequences was allowed). Assembly of cleaned reads was performed using CAP3 (Huang and Madan 1999) and contigs were aligned to the set of reference UCEs using LASTZ Release 1.02.00 (Harris 2007). Contigs that aligned with more than one reference UCE and different contigs that aligned with the same reference UCE were filtered out using Geneious 8.1.8. (https://www. geneious.com).

UCEs for which sequences were available for more than 50% of the taxa were kept in the next steps of the analysis. Alignments were performed with MAFFT v7.245 (Katoh and Standley 2013) (-linsi option). Ambiguously aligned blocks were removed using Gblocks_0.91b with relaxed constraints (-t = d -b2 = b1 -b3 = 10 -b4 = 2 -b5 = h) (Talavera and Castresana 2007). The final data set was analysed using supermatrix approaches based on maximum likelihood (ML) as implemented in raxmlHPC-PTHREADS-AVX (Stamatakis 2014) (version 8.2.11) and IQ-TREE v1.6.7 (Nguyen et al. 2015) and the coalescent-based summary method implemented in ASTRAL-III v5.6.3 (Zhang et al. 2018). For the RAxML analysis, a rapid bootstrap search (100 replicates)

followed by a thorough ML search (-m GTRGAMMA) was performed. For the IQ-TREE analysis, 20 independent ML searches were conducted using the best-fit substitution model automatically selected by ModelFinder (Kalyaanamoorthy et al. 2017) (candidate tree set for each search = 98 parsimony trees + BIONJ tree with only the 20 best initial trees retained for NNI search). Branch supports were assessed with ultrafast bootstrap (Minh et al. 2013) and SH-aLRT test (Guindon et al. 2010) (1000 replicates) as well as standard non-parametric bootstrap (100 replicates). A bootstrap convergence test was performed with RAxML using the extended majority-rule consensus tree criterion (Pattengale et al. 2010). Individual trees were inferred from each UCE using RAxML (-f a -x 12,345 -p 12,345 -# 100 -m GTRGAMMA) and used as input for the ASTRAL analysis. To improve accuracy in the ASTRAL analyses, nodes within loci that had BP support <10 were collapsed in individual gene trees (Zhang et al. 2018) with the perl script AfterPhylo.pl (Zhu 2014). Node supports were evaluated with local posterior probabilities (local PP). Summary statistics were calculated using AMAS (Borowiec 2016). Tree annotation was performed with TreeGraph 2.13 (Stöver and Müller 2010).

Computational resources

Analyses were performed on a Dell PowerEdge T630 server with two 10-core Intel(R) Xeon(R) CPUs E5-2687W v3 @ 3.10 GHz and on the Genotoul-bioinfo Cluster (INRA, Toulouse).

Results and discussion

UCE data set

The analysed data set included 99 taxa and 879 UCEs. Taxa were represented by 110–826 UCEs (median 688, Table S1; Figure 1). Ten taxa had more than 50% missing UCEs, and only two had more than 80% missing UCEs. The alignment cleaned with Gblocks contained 270,760 bp, 68.2% of which were parsimony informative. The percentage of missing data (due to capture failure in some taxa) was 24.7%, the percentage of gaps (that can either be created while aligning full-length UCEs or result from the capture of incomplete UCEs) was 8.6% and the GC content was 42.4%.

Phylogenetic inference

ModelFinder selected TIM+F + I+ G4 as the best-fit model for the concatenated data set (Bayesian Information Criterion). The 20 independent ML searches conducted with IQ-TREE converged to the same topology (Figure S1). The bootstrap convergence test indicated that stable support values were reached after 50 replicates of the standard non-parametric bootstrap. IQ-TREE and RAxML inferred identical and well-resolved topologies (Figures S1–S3). Fifteen nodes on 97 received standard bootstrap support values lower than 100 (Figure S1). Comparison of standard BP values (IQ-TREE), rapid BP values (RAxML) and ultrafast bootstrap values (UFBoot, IQ-TREE) did not indicate that either rapid BP or UFBoot largely overestimated node support (Figure S4). In two cases, rapid BP were 100 and UFBoot + SH-aLRT provided high support according to confidence thresholds
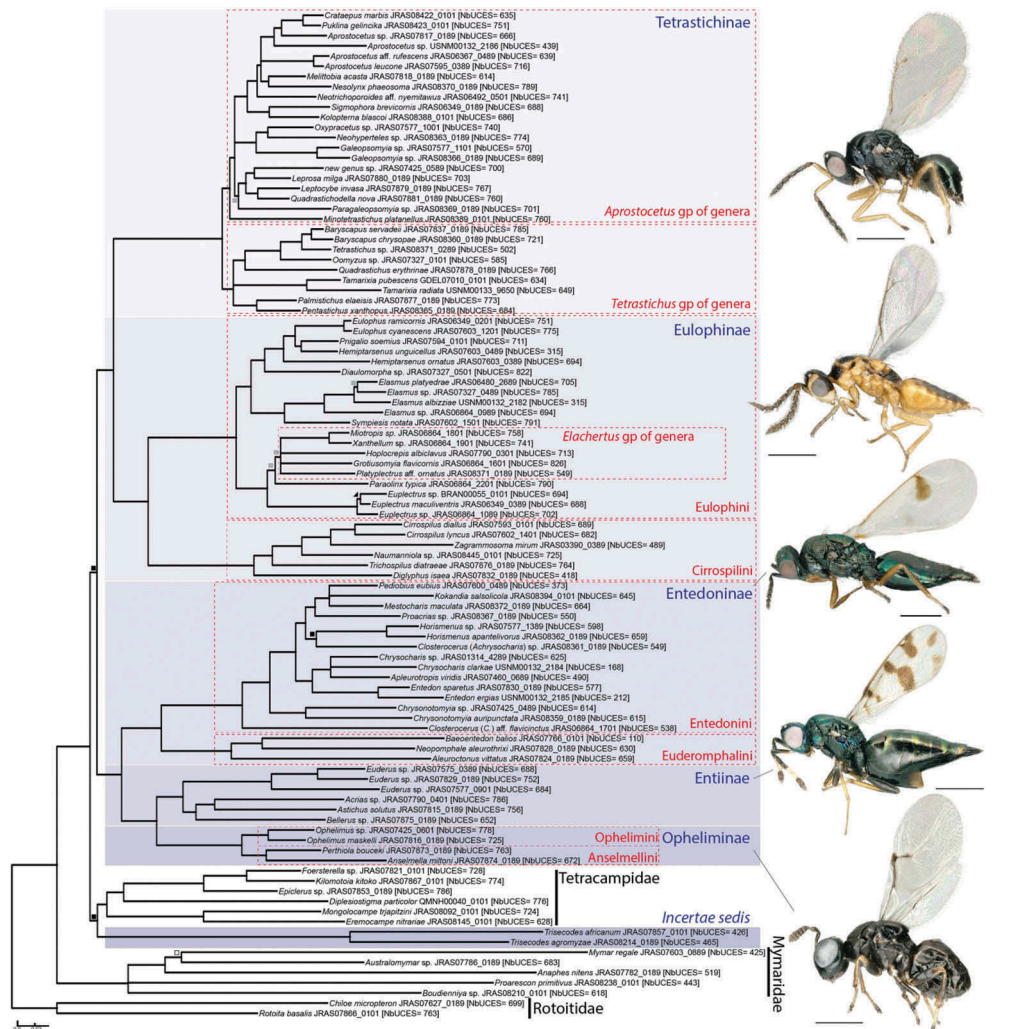
Figure 1. Phylogenetic relationships among eulophids inferred from 879 UCEs.

The IQTREE tree is used as template, and the number of UCEs analysed for each sample is given in brackets. Nodes that were not recovered by both approaches (supermatrices ML – RaxML/IQTREE and gene tree reconciliation ASTRAL) are collapsed. All nodes were highly supported (RAxML rapid BP >95; IQTREE SH-aLRT >80/UFBoot >95; IQTREE standard BP >95; ASTRAL local PP >0.8) unless specified with symbols. White squares indicate nodes that were observed in all trees but did not receive significant support (RAxML BP <95; IQTREE SH-aLRT <80/UFBoot <95; IQTREE standard BP <95; ASTRAL local PP <), grey squares indicate nodes supported only by ASTRAL (RAxML BP <95; IQTREE SH-aLRT <80/UFBoot <95; IQTREE standard BP <95; ASTRAL local PP >0.8), black squares indicate nodes supported only by ML approaches (RAxML BP >95; IQTREE SH-aLRT >80/UFBoot >95; IQTREE standard BP >95; ASTRAL local PP <0.8) and black triangles indicate nodes supported only by IQTREE with non-standard bootstrapping approaches (RAxML BP <95; IQTREE SH-aLRT >80/UFBoot >95; IQTREE standard BP <95; ASTRAL local PP <0.8). Complete RAxML, IQTREE and ASTRAL trees are available in Figures S1–S3, S5. Photos ©J.-Y. Rasplus. Scale bars = 500 μm.

mentioned in the IQ-TREE manual (i.e. SH-aLRT >80/UFBoot >95) when standard BP were only 88 and 78. In one case, UFBoot + SH-aLRT provided high support when standard BP and rapid BP were only 76 and 80, respectively. For all but three nodes, values of rapid BP were equal or lower than standard BP values. The ML and ASTRAL (Figure S5) trees were globally congruent. The consensus tree of these two approaches is presented in Figure 1.

Only seven nodes that received poor statistical supports in both approaches (ML and ASTRAL) were in conflict (represented by polytomies in Figure 1). With the exception of the genus *Trisecodes* that clustered with the outgroups (sister to Tetracampidae), Eulophidae was recovered monophyletic (with high support only in the ML approach). All subfamilies and tribes were also recovered monophyletic with high support. The placement of the few specimens (*n* = 3) with a high proportion of missing data (<250 loci sequenced) agreed with morphology.

Our phylogenetic results support two major clades within Eulophidae: a clade formed by Tetrastichinae + Eulophinae and a clade containing Entedoninae sister to Entiinae + Opheliminae. This result corroborates the hypothesis of relationships among subfamilies proposed by Burks et al. (2011) and Munro et al. (2011).

In contrast to previous phylogenetic studies that have reported high bootstrap support (>90%) for approximately half of the suprageneric nodes, our analyses recovered high support for about 90% of the suprageneric nodes (standard bootstrap ≥99). Our analyses provided strong support for a number of notable relationships within the subfamilies of Eulophidae. However, two clades had less supported relationships: (1) the *Elachertus* group of genera and (2) the *Aprostocetus* group of genera. Both clades show short internode branches that may be indicative of rapid diversification. Overall, these results highlight the power of UCEs to reconstruct a robust phylogenetic hypothesis for the family.

Trisecodes, the only eulophid with three segmented tarsi in female (Delvare and Lasalle 2000), was previously classified as *incertae sedis* within Eulophidae (Burks et al. 2011). Our analysis that includes the neotropical *T. agromyzae* Delvare and LaSalle, 2000, and the afrotropical *T. africanum* Gumovsky, 2014, supports other earlier studies that have placed *Trisecodes* as distantly related to other Eulophidae (Burks et al. 2011; Munro et al. 2011; Heraty et al. 2013). *Trisecodes* is recovered as sister to Tetracampidae by both ML and ASTRAL approaches, but this relationship is only supported by ML approaches. Outgroup sampling being reduced, the most cautious conclusion at the time of this study is that *Trisecodes* does likely not belong to Eulophidae; however, its correct classification still awaits a large-scale phylogenomic hypothesis for Chalcidoidea.

Anselmella and *Perthiola*, two Australian genera belonging to the tribe Anselmellini (Bouček 1988; Reina and LaSalle 2005), formed a strongly supported clade sister to the tribe Ophelimini. Consequently, the unplaced tribe Anselmellini belongs to the recently recognised subfamily Opheliminae (Burks et al. 2011), a result already suggested by Munro et al. (2011). No tribal-level classification has yet been proposed for Entiinae, probably because the sampling of this subfamily has always been limited in previous phylogenetic studies.

Within Entedoninae, Euderomphalini (parasitoids of whiteflies) is recovered sister to Entedonini, a placement corroborated by the results from Burks et al. (2011). The only species of the genus *Closterocerus* subgenus *Closterocerus* included in this study was recovered sister to all other Entedonini, a result also similar to Burks et al. (2011), yet with higher support. The second species of *Closterocerus*, belonging to the subgenus *Achrysocharis*, was nested within Entedonini and recovered sister to *Horismenus* with high statistical support in ML analyses. This result highlights the difficulty to reliably define some entedonine genera based only on morphology. *Chrysonotomyia* was recovered sister to all other Entedonini, and the remaining Entedonini was further subdivided into

two groups of genera: (1) *Entedon* + *Chrysocharis* and *Apleurotropis* and (2) *Pediobius* + *Kokandia* + *Mestocharis* sister to *Proacrias* and *Horismenus* + *Closterocerus* (*Achrysocharis*). The first clade is well defined by one synapomorphy: the transverse carina on lateral pronotum (sometimes appearing as a semicircular plica) (Gumovsky 2002; Burks et al. 2011).

Eulophinae is traditionally subdivided into two tribes – Cirrospilini and Eulophini – which is an arrangement confirmed by our results. Within Cirrospilini, the relationships observed between the genera globally match those observed by Ubaidillah et al. (2003) using 56 morphological characters. There are three clades within the tribe Eulophini: one corresponds to the *Elachertus* group of genera, another groups *Sympiesis* and *Elasmus* and the third includes all other genera of Eulophini included in our study. Our results confirmed the placement of *Elasmus* nested within Eulophinae. Gauthier et al. (2000) synonymised the family Elasmidae with Eulophidae and *Elasmus* appeared sister to *Sympiesis* in our topologies. This relationship needs to be confirmed as it is not supported by morphology, which instead groups *Sympiesis* with *Pnigalio* and *Hemiptarsenus*. *Hemiptarsenus* is recovered paraphyletic with the two species included in our analysis forming a grade. This result confirms morphological analysis that also strongly suggested the para- or polyphyly of the genus (Burks 2003), with some species found to render *Sympiesis* paraphyletic and some others most closely related to *Pnigalio*.

Tetrastichinae was subdivided into two strongly supported clades that match the hypothesis proposed by Graham (1987): (1) the *Aprostocetus* group of genera and (2) the *Tetrastichus* group of genera. In the future, these entities may deserve a tribal status if analyses including a larger sampling of the genera of Tetrastichinae confirm this dichotomy. Within the *Aprostocetus* group of genera, our analyses recovered *Minotetrastichus* sister to all other genera. This result contradicts a previous hypothesis that suggested that *Minotetrastichus* was closely related to *Aprostocetus* (Graham 1987). This genus differs from other genera of the group by having extremely small propodeal spiracles. The *Aprostocetus* group of genera is further subdivided into three well-supported clades: (1) an Australian clade of gall-making tetrastichines sister to the New World genus *Paragaleopsomyia*; (2) a New World clade of genera containing *Galeopsomyia* and related genera and (3) a clade of genera closely related to *Aprostocetus*. These genera mostly occur in the Old World, but some are sub-cosmopolitan. Within this clade, *Puklina* + *Crataepus* render the genus *Aprostocetus* paraphyletic. This may raise questions about the validity of these genera as they may only represent derived species groups of *Aprostocetus*. However, this may also suggest that several species groups of *Aprostocetus* as presently defined deserve generic status.

Conclusion

We present the first phylogenomic hypothesis for Eulophidae using UCEs. At higher levels (tribes and subfamilies), our results were consistent with previous analyses based on morphology and a set of a few molecular markers. However, our analysis provides high support for most nodes, including intergeneric relationships for which morphology and previous molecular markers failed to provide robust resolution. Therefore, UCEs may represent ideal molecular markers for inferring a dense phylogeny of Eulophidae at a global level. The taxonomy of eulophids has been hampered by their homogenous

morphology. In several groups of eulophids (Entedoninae, Tetrastichinae), the generic delimitation based on morphology has been challenging due to the difficulty to identify reliable characters. Among the several hundred genera of eulophids, only a few have been explicitly defined on the basis of synapomorphies. Given the lability of most morphological characters used to define genera, reconstructing a thorough phylogenetic hypothesis with dense sampling may help testing their monophyly and identifying useful characters for future generic classifications.

The current lack of a robust phylogenetic framework for Eulophidae is also a major drawback for many other areas of research, such as historical biogeography and the evolution of life-history traits. Indeed, eulophids exhibit a wide variety of biology (phytophagous, primary idiobiotic and koinobiotic parasitoids and hyperparasitoids) and a large spectrum of hosts, with multiple shifts between biologies and hosts. Few studies have examined the evolution of host ranges within Eulophidae. Identifying the most likely evolutionary pathways that led to the current disparity of Eulophidae lifestyles will, of course, depend on our ability to improve our knowledge of host-eulophid associations but also on our ability to produce a thoroughly documented and well-supported phylogeny of the family, which now appears feasible.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability

Fastq raw reads are available as NCBI Sequence Read Archives (BioSamples IDs are listed in Table S1). The concatenated data set and newick tree files are available from Zenodo (https://doi.org/10.5281/zenodo.3753257).

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