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Phylogenomic analysis of *Stylops* reveals the evolutionary history of a Holarctic Strepsiptera radiation parasitizing wild bees

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ABSTRACT

Holarctic *Stylops* is the largest genus of the enigmatic insect order Strepsiptera, twisted winged parasites. Members of *Stylops* are obligate endoparasites of *Andrena* mining bees and exhibit extreme sexual dimorphism typical of Strepsiptera. So far, molecular studies on *Stylops* have focused on questions on species delimitation. Here, we utilize the power of whole genome sequencing to infer the phylogeny of this morphologically challenging genus from thousands of loci. We use a species tree method, concatenated maximum likelihood analysis and Bayesian analysis with a relaxed clock model to reconstruct the phylogeny of 46 *Stylops* species, estimate divergence times, evaluate topological consistency across methods and infer the root position. Furthermore, the biogeographical history and coevolutionary patterns with host species are assessed. All methods recovered a well resolved topology with close to all nodes maximally supported and only a handful of minor topological variations. Based on the result, we find that included species can be divided into 12 species groups, seven of them including only Palaearctic species, three Nearctic and two were geographically mixed. We find a strongly supported root position between a clade formed by the *spreta*, *thwaitesi* and *gwynanae* species groups and the remaining species and that the sister group of *Stylops* is *Eurystylops* or *Eurystylops* + *Kinzelbachus*. Our results indicate that *Stylops* originated in the Western Palaearctic or Western Palaearctic and Nearctic in the early Neogene or late Paleogene, with four independent dispersal events to the Nearctic. Cophylogenetic analyses indicate that the diversification of *Stylops* has been shaped by both significant coevolution with the mining bee hosts and host-shifting. The well resolved and strongly supported phylogeny will provide a valuable phylogenetic basis for further studies into the fascinating world of Strepsipterans.

1. Introduction

"*One of the most curious of all insects is your Stylops*" wrote Professor William Dandridge Peck at Harvard University to Reverend William Kirby in Suffolk, England in a letter dated 21st of September 1809 (Kirby, 1813). Four years later William Kirby described the new insect order Strepsiptera named after the rudimentary and twisted anterior pair of wings (Kirby, 1813). The biology, life cycles and phylogenetic placement within insects of this new order have perplexed biologists ever since (Cook, 2014; Kathirithamby, 1989, 2018; Pohl & Beutel, 2013). Known by the vernacular name twisted winged parasites, they were referred to as "*twisted parasites from outer space*" in a commentary in Science (Proffitt, 2005). The phylogenetic belonging of this enigmatic order has finally been settled as the sister group of Coleoptera thanks to the power of genomic data (Boussau et al., 2014; Misof et al., 2014; Niehuis et al., 2012), but the group continue to baffle in many other ways (Bravo et al., 2009; Johnston et al., 2004; Kathirithamby, 2018; Peinert et al., 2016).

Strepsiptera is a small insect order with around 600 valid species in ten extant families (Cook, 2019; Kathirithamby, 2018, 2021). More than

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a quarter of all species belong to family Stylopidae, "*the peak of strepsipteran diversification*" (Pohl & Beutel, 2008), dominated by the genus *Stylops* Kirby, 1802. All species in Stylopidae are bee-parasites and members of *Stylops* are restricted to *Andrena* Fabricius, 1775 mining bees (family Andrenidae) (Kathirithamby, 2018). The biology and life cycle of *Stylops* is representative of the large majority of Strepsipterans. Both sexes have a free-living and host-seeking first instar stage, followed by endoparasitic larval stages inside the body of the host. At the end of the last larval instar stage, both sexes extrude their anterior body regions through the host cuticle. Males pupate in the partly extruded position whereas females have a reduced pupal stage (Erezyilmaz et al., 2014; Löwe et al., 2016). Winged adult males leave the host to live for only a few hours to mate, while the larviform females remain as endoparasites in the host, releasing pheromones to attract males and alter the host's behaviour to facilitate reproduction (Cvačka et al., 2012; Straka et al., 2011). The distribution of *Stylops* is constrained to the Holarctic region (Kathirithamby, 2018) as is largely the host genus *Andrena* (Pisanty et al., 2022). The estimated number of valid species in the genus ranges from 68 (Straka et al., 2015) to 105 (Cook, 2019), depending on what is believed to be the host range for a given species. Based on recent molecular studies, *Stylops* species tend to parasitize few closely related host species, often belonging to the same *Andrena* subgenus, which supports the more conservative species estimate (Jůzová et al., 2015, Lähteenaro et al., 2024).

Despite *Stylops* being the most diverse extant genus of Strepsiptera, no fossil record is associated with the genus (Kathirithamby, 2018; Kogan & Poinar, 2020). The rarity of Strepsiptera amber inclusions likely stems from their life-history traits. Owing to the short reproduction-focused lives of adult males, the probability of them getting caught in tree resin is quite low. One male Strepsiptera in Dominican amber was erroneously assigned to *Stylops* (Kogan & Poinar Jr, 2010) and subsequently recognized as *Palaeomyrmecolax* Kulicka, 2001 in family Myrmecolacidae (Kogan et al., 2015). Another male Stylopidae fossil from the monotypic genus *Jantarostylops* Kulicka, 2001 was reported from Baltic amber (Kulicka, 2001). However, this is potentially a misidentification as well, due to the resemblance between antennal structures of Myrmecolacidae and Stylopidae. Lastly, there is a Dominican amber inclusion of a stylopised halictid bee with an empty male puparium, potentially belonging to *Halictoxenos* Pierce, 1909 in Stylopidae (Poinar, 2004) but not possible to identify with certainty. Hence, the timeline of the evolutionary history of bee-parasitic Strepsiptera is largely reliant on more distant fossil or indirectly through the evolutionary history of their hosts.

The higher-level phylogeny within Strepsiptera has been studied using both cladistic analyses of morphological characters (Pohl, 2002; Pohl et al., 2021; Pohl & Beutel, 2005, 2008) and model-based analyses with a small number of molecular markers (McMahon et al., 2011). Stylopidae is strongly supported as monophyletic and are united by parasitizing bees in multiple closely related families (Andrenidae, Halictidae, Colletidae and Melittidae). The relationship between the seven genera in Stylopidae is not resolved and there are various hypotheses as to the sistergroup of *Stylops* (Bohart, 1941; Ulrich, 1964; Kinzelbach, 1971, 1990; Pohl & Beutel, 2005; Jůzová et al., 2015; Benda et al., 2020; Pohl et al., 2021). To our knowledge, there has been no previous study focusing on the intrageneric phylogeny of *Stylops* species. Studies that have included some kind of phylogenetic analyses and more than two representatives of *Stylops* have been either geographically restricted single gene trees of mitochondrial COI (Hoffmann et al., 2023; Smit et al., 2020) or have concentrated on questions of species delimitation within *Stylops* (Jůzová et al.,2015; Lähteenaro et al., 2024). Lähteenaro et al.'s (2024) species delimitation study using genomic data was restricted to Western Palaearctic taxa. Jůzová et al. (2015) used two mitochondrial and one nuclear gene and had a Holarctic scope in sampling, but terminal names consisted of unidentified *Stylops* sample codes with only host IDs for the purpose of testing hypotheses of species delimitation in relation to host breadth. In addition, Jůzová et al., (2015,p. 235) concluded "*Stem branching within the genus Stylops remains completely unresolved*" due to lack of node support. Smit et al. (2020) DNA barcoded *Stylops* species from the Netherlands and provided an outgroup-rooted neighbour-joining COI gene tree to characterize the local Strepsiptera fauna. Hoffmann et al. (2023) studied the single species *Stylops ater* Reichert, 1914, but provided a maximum likelihood COI gene tree that also included some other species as outgroups from Jůzová et al. (2015) and Smit et al. (2020).

One explanation for the lack of other previous studies may be the difficulties related to sample acquisition as well as cryptic species and overly broad species concepts. Adult *Stylops* males, which have more distinct interspecific characters, are rarely encountered due to their short life span, whereas *Stylops* females are larviform with few structural differences between species, even causing Kinzelbach (1978) at the time to synonymize all Western Palaearctic species. At least in Europe this view dominated during the golden age of cladistic morphological analyses and there was hence no phylogeny to infer, at least among European taxa. Today we know *Stylops* is the most diverse genus of Strepsiptera also in Western Palaearctic (Jůzová et al., 2015; Lähteenaro et al., 2024) and it is high time to focus on the intrageneric relationship of "the peak of Strepsiptera diversification" using available sequencing technology.

In this study, we investigate the phylogenetic relationships among *Stylops* species using whole genome sequencing. We specifically aim to i) infer the position of the root within *Stylops*, ii) define natural species groups, iii) reconstruct ancestral distributions with a time window (dating), and iv) test for coevolution with a published phylogeny of the hosts. Apart from the inherent coevolutionary potentials in a host-parasite system, the higher phylogeny of Strepsiptera to some degree follows the insect host phylogeny. Mengenillidia are parasites on apterygote insects, Stylopidia on pterygote insects. Corioxenidae parasitizes hemimetabolous insects whereas the ancestor of Stylopiformia was reconstructed as a parasite on holometabolous insects (McMahon et al., 2011). We hypothesize that this macroevolutionary congruence could be mirrored by similar coevolutionary patterns also on the intrageneric level within *Stylops*

2. Materials and methods

2.1. Taxon sampling, sequencing, and data assembly

In order to cover the entire distribution area of the genus *Stylops*, we expanded the genomic data set used by Lähteenaro et al. (2024) to also include Nearctic species (Table S1). A total of 46 species were included: 18 Nearctic (newly sequenced), 22 Western Palaearctic, out of which two occur also in Eastern Palaearctic, and two restricted to Eastern Palaearctic (Fig. 1). The taxonomy used here follows Straka et al. (2015) except for when updated in light of Lähteenaro et al (2024). We used an integrative species identification method employing both the host association information and morphology of the sampled females. If the host species could not be identified reliably, COI was extracted from the *Stylops* assembly with Alibaseq v1.2 (Knyshov et al., 2021) using available COI *Stylops* sequences in GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) as baits. The species were assigned based on the best hit from nucleotide BLAST (Altschul et al., 1990; Zhang et al., 2000) with a minimum identity cut off (99.5 %) and the morphology of the specimen was compared with species descriptions to confirm the match (literature listed in Table S1).

We included seven outgroup species: *Mengenilla moldrzyki* Pohl, Niehuis, Gloyna, Misof & Beutel, 2012 (Mengenillidae), *Triozocera* sp. Pierce, 1909 (Corioxenidae) and *Xenos vesparum* Rossius 1793 (Xenidae) from the data set of McKenna et al. (2019), and *Eurystylops oenipontana* Hofeneder, 1949 (Stylopidae), *Halictoxenos tumulorum* Perkins, 1918 (Stylopidae), *Halictoxenos spencei* Nassonov, 1893 and *Kinzelbachus friesei* (Hofeneder, 1949) (Stylopidae) from Straka et al. (in prep.). *Mengenilla moldrzyki,* the only representative not part of suborder

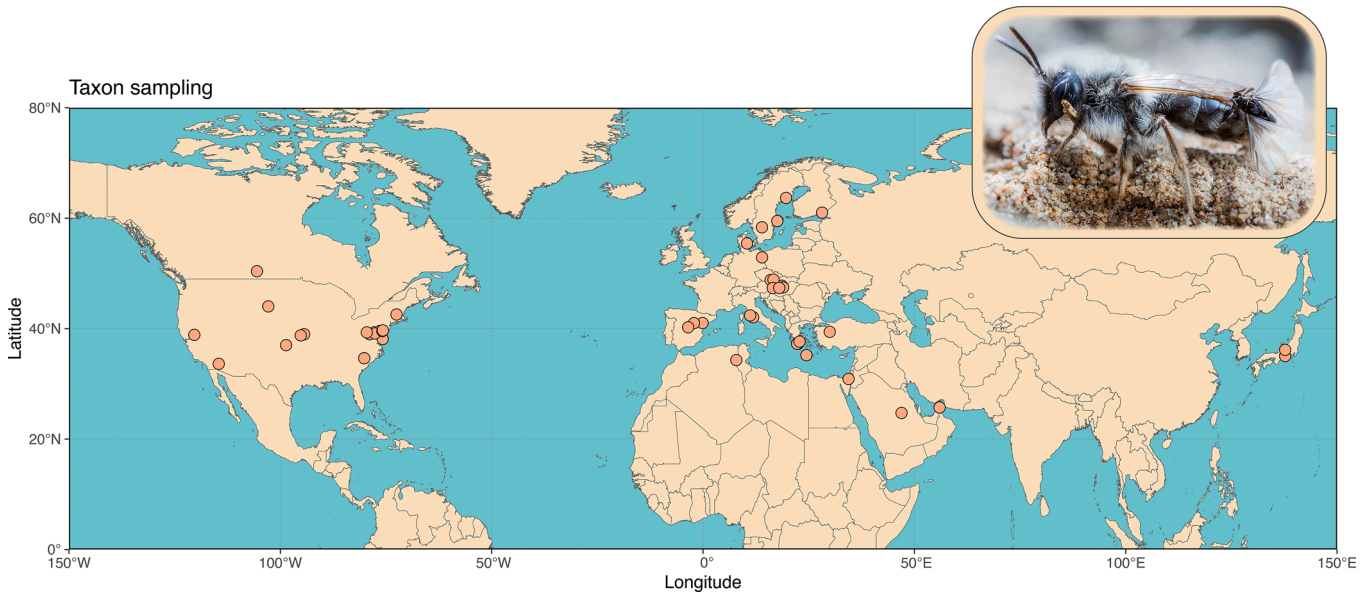

Fig. 1. Holarctic map of sampling locations for the phylogenetic dataset of *Stylops*. Inset photo of *Stylops ater* (male) and its host *Andrena vaga* (Photo: Johan Lind/N).

Stylopidia, was used to root the tree.

DNA was extracted from *Stylops* females, out-dissected from the host abdomen, using QIAamp DNA Micro kit (Qiagen, Inc). Nextera DNA flex libraries were built at the Science for Life Laboratory (SciLifeLab), Stockholm, National Genomics Infrastructure (NGI) Sweden, for samples with higher DNA concentration. For samples with low DNA concentration, illlumina libraries were prepared following a special protocol aimed for degraded DNA (Irestedt et al., 2022). All samples were sequenced in SciLifeLab using the lllumina NovaSeq sequencing platform. The whole genome sequencing data was pre-processed and assembled with Nf-core/eager v2.4.0 pipeline (Yates et al., 2021). For further details on lab protocols and sequence assembly, see Lähteenaro et al. (2024). The produced assemblies were searched for 3,913 nuclear orthologous genes acquired from the data set of McKenna et al. (2019), which contained four Strepsiptera species including one *Stylops*. The genes of the four taxa were extracted from the McKenna et al. dataset with grepfasta (https://github.com/nylander/grepfasta) and gaps and sequences shorter than 100 bp were removed with fastagap (https://github.com/nylander/fastagap). We used the extracted genes as nucleotide baits to extract the single best hit regions from our nucleotide assemblies with Alibaseq v1.2 (Knyshov et al., 2021). The produced fasta files were processed with ATPW – Align-and-Trees-Parallel-Workflow (https://github.com/nylander/Align-and-trees-parallel-workflow). In it, the recovered orthologous genes were aligned with Mafft v.7.490 (Katoh et al., 2002; Katoh & Standley, 2013) and the multiple sequence alignments (MSA) were checked for formatting errors in RaxML-NG (Kozlov et al., 2019) and phylogenetically informative regions were selected with BMGE (-h 0.5 -g 0.2 -b 5) (Criscuolo & Gribaldo, 2010). We used ParGenes (Morel et al., 2019) for conducting model selection and gene tree inference in parallel for all MSAs. First maximum likelihood trees were inferred with RAxML-NG using a fixed model (GTR + G8 + F) to identify and remove outlier branches with TreeShrink (Mai & Mirarab, 2018). The filtered data was re-aligned and ParGenes was run again including a model test to select a best-fit model of evolution for each MSA based on the Bayesian information criterion (BIC). The produced gene trees and MSAs were used in subsequent inferences. As multiple studies have indicated that saturation in the third codon positions in alignments may influence inferred relationships when analysing nucleotide data (eg. Breinholt & Kawahara, 2013; Kulkarni et al., 2021), we generated saturation plots based on uncorrected-to-corrected distances and uncorrected distances against tree-based distances as in Klopfstein et al. (2013) to assess the saturation levels in our data (Fig. S2).

2.2. Phylogenetic analysis and divergence dating

Three types of phylogenies were inferred: a species tree (ST) under the multi-species coalescent (MSC) model in ASTRAL-III v.5.6.3 (Zhang et al., 2018), a Maximum Likelihood (ML) tree in IQ-TREE2 v.2.2.0 (Minh et al., 2020) and a relaxed clock tree using Bayesian inference (BI) in BEAST2 (Bouckaert et al., 2014). Outgroups were included in the ASTRAL-III and IQ-TREE2 inferences to root the tree. The species tree in ASTRAL-III was inferred to account for incomplete lineage sorting (ILS) which standard maximum likelihood analyses ignores. The ASTRAL-III species tree was constructed from the inferred gene trees acquired from RaxML-NG (Kozlov et al., 2019), which included model selection. As genetic distances involving outgroups (but not for the ingroup alone) showed signs of saturation in the saturation plots of 3rd codon positions (Fig. S2), we additionally ran ASTRAL-III for gene trees inferred from MSAs with third positions excluded (nt12), including the use of a gene tree support weighting scheme due to the expected increase in gene tree estimation error (Zhang & Mirarab, 2022). This was specifically done to examine if it had any effect on the inferred of a sister-group to *Stylops*.

While accounting for ILS, species tree methods may be sensitive to gene tree estimation error (GTEE) (Gatesy & Springer, 2014; Roch & Warnow, 2015; Springer & Gatesy, 2016). If GTEE is high and level of ILS is low, maximum likelihood analyses can be superior (Mirarab et al., 2014). To assess the sensitivity of the inference to the length of loci, loci shorter than 100 bp (expected to have a higher degree of GTEE on average) were excluded and ASTRAL-III was run again. The ML tree was inferred from a concatenated supermatrix of all MSAs, and again with only MSAs longer than 100 bp, to reduce the negative impact of sequence length heterogeneity to the accuracy of ML methods (Smirnov & Warnow, 2021). We estimated branch support with ultrafast bootstrap (UFBoot, -bb 1000) using an additional optimization step, ("-bnni" nearest neighbour interchange optimization), which can reduce the risk of overestimating branch support (Hoang et al., 2018). Additionally, SH-like approximate likelihood ratio test (SH-aLRT, -alrt 1000) (Guindon et al., 2010) was performed as it is less likely to underestimate support on short branches compared with bootstrapping (Alfaro et al., 2003; Guindon et al., 2010). ModelFinder (Kalyaanamoorthy et al., 2017) was used for model selection and for estimating the best partitioning scheme, which were then used for the tree construction (-m MFP + MERGE) (Nguyen et al., 2015). A relaxed clustering algorithm was used to lower

the computational load (-rcluster 10). The ML inference was repeated five times to check that the optimal tree was found. Again, to evaluate if saturation in third codon positions had any effect on outgroup resolutions, we repeated the ML inference with third positions excluded (nt12), with original (GTR + F + R6) and reoptimized substitution model (GTR + F + I + R6).

Outgroups were not included in the Bayesian inference in order to test the root placement inferred by the outgroup method by using a molecular clock model instead. Strepsiptera are known for their high rates of sequence evolution (Boussau et al., 2014; McMahon et al., 2011) and large distances between ingroup and outgroups may lead to random rooting (e.g. Graham et al., 2002; Tarrío et al., 2000; Wheeler, 1990). From our analysis and inclusion of outgroup taxa in ML, it appears that whichever outgroup is used, it will constitute a very long terminal branch compared with the branches within the *Stylops* diversification. This necessitates implementation of an independent method for root position assessment. Three data sets were used for the BI to test the sensitivity of the result to missing data: one with 100 % taxon occupancy (TO100), one with more than 50 % taxon occupancy (TO50) and one with no additional filtering in the gene selection step for the missing data in terms of taxon occupancy (NF). A subset of 150 loci was selected from each data set using recommendations from the SortaDate package (Smith et al., 2018). SortaDate is a pipeline for phylogenomic subsampling that selects informative and low-variance genes suitable for being analysed with Bayesian methods using clock models. This is a practical necessity since it is not feasible to analyse entire genomic datasets with Bayesian Markov chain Monte Carlo methods. SortaDate uses three gene properties for the gene-selection: root-to-tip variance, tree-length and bipartitions. Out of the three, root-to-tip variance has the largest impact on the selection (Mongiardino Koch, 2021). Root-to-tip variance is an indication of clock-likeness and using clock-like genes can reduce errors associated with model-misspecifications (Smith et al., 2018). Each subset of genes (NF/TO50/TO100) was concatenated with the program pxcat in phyx and converted to nexus format with the program phynex in phyx producing a final matrix of 40,018/40,371/48,531 characters (Brown et al., 2017). Additionally, to test the effect of subsampling based on SortaDate, 150 loci were selected randomly from each dataset (NF, TO50, TO100) and processed in a similar manner.

The three data sets were tested for the suitability of a strict molecular clock model in MrBayes v.3.2.7 (Ronquist et al., 2012, 2020). Based on the test results, an optimized relaxed clock (Douglas et al., 2021; Drummond et al., 2006) was used for the BI inference in BEAST2 (Bouckaert et al., 2014). The best fit models for the data sets were estimated with ModelFinder (Kalyaanamoorthy et al., 2017) and these were used in BEAST2 as substitution models. A Yule model (Gernhard, 2008) with a log-normal birth rate was used as a tree prior. We ran the analysis for 30 million Markov chain Monte Carlo generations and discarded the first 10 % as burn-in based on assessment in Tracer v.1.7.2 (Rambaut et al., 2018). Each run was repeated and the results of the two independent runs were combined with LogCombiner v.2.7.3 (Bouckaert et al., 2014) before selecting a maximum clade credibility (MCC) tree with median node heights using TreeAnnotator v.2.7.3 (Bouckaert et al., 2014). Trees were visualized using FigTree v.1.4.4 (Rambaut, 2018). All procedures were repeated for the randomly selected loci sets.

A topology test with four-cluster likelihood mapping (FcLM, Strimmer & von Haeseler, 1997) was performed for a backbone node with conflicting signal between the tree inference methods. The FcLM was done in IQ-TREE2 with automatic best-fit model selection (-m TEST). The clusters were assigned based on the conflicting node and 1,500 quartets were drawn randomly (-lmap 1500). Outgroups were excluded from the analysis.

The dating analyses were performed with BEAST2 using TO50 loci dataset and same settings as before, except an age prior was added to the MRCA of *Stylops*. Due to the lack of reliable fossil evidence, we used secondary calibrations from two dated phylogenies of *Andrena* bees (Cardinal et al., 2018; Pisanty et al., 2022), since it is unlikely for the divergence time of host-specific parasites to be older than that of their hosts. Cardinal et al. (2018) used a set of 34 reviewed and confidently placed fossil taxa to date the phylogeny of bees (Anthophila), *Andrena* being represented by three species. The deep sampling of *Andrena* species in Pisanty et al. (2022) provides a higher resolution of stylopized taxa while being based on the calibrations in Cardinal et al. (2018). The lower limit of *Andrena* stem age is 38 Ma (Cardinal et al., 2018) and upper limit of known stylopized *Andrena* lineage 16 Ma (Pisanty et al., 2022). Thus, we assigned the MRCA node a normal prior distribution (mean = 27.7, Std = 6.0), which gives 95 % of ages between 16 and 38 Ma. The MCC tree was calculated from four independent runs similar to above.

2.3. Ancestral biogeographic range estimation

We used the R package BioGeoBEARS (Matzke, 2013, 2014) to infer the biogeographic history of *Stylops* by estimating the most likely model of geographic range expansion on the dated MCC tree obtained from BEAST2. We applied a modified version of a script provided by the author of the program (http://phylo.wikidot.com/biogeobears#script, accessed 31.5.2023) to run the analysis. The included models were Dispersal-Extinction-Cladogenesis or DEC (Ree & Smith, 2008), DIVALIKE, a likelihood version of Dispersal-Vicariance-Analysis (Ronquist, 1997), and BAYAREALIKE, a likelihood version of BayArea (Landis et al., 2013). Additionally, we included founder-event speciation (+J), into each of the models. Even though the use of parameter + J has faced criticism (Ree, & Sanmartín, 2018), we decided to include it into our analyses in the light of justifications provided by Matzke (2022) as it may provide improvements in model fit. As parasites have higher frequency of founder event speciation (Hoberg & Brooks; Huyse et al., 2005), including + J is likely to increase the model fit and hence increase the accuracy of inferences. The fit of these six models on our data was assessed with Akaike information criterion and Log likelihood scores (LnL). Due to the controversy around the + J parameter, we also selected the best model among models without it. Biogeographical areas were defined as Western Palaearctic (W), Eastern Palaearctic (E) or Neartic (N) based on extant distributions of *Stylops* species. The species were limited to occur only in two areas, as none of the extant species inhabit all three areas (max_range_size = 2).

2.4. Cophylogenetic analyses

The cophylogenetic congruence between clades of *Stylops* and *Andrena* was assessed with Procrustean Approach to Cophylogeny (PACo) (Balbuena et al., 2013) implemented in R statistical software environment v.4.2.2 (R Core Team, 2022) with package paco v.0.4.2 (Hutchinson et al., 2017). PACo is a global-fit method (see Dismukes et al., 2022 for a review) which tests the dependency of one phylogeny on the other, using Procrustean superimposition on patristic distance matrices of the host and parasite trees. By randomly assigning hosts to parasites during a permutation procedure, PACo produces a significance level of the global fit. The contribution of individual host-parasite links to overall cophylogenetic signal can be assessed either through jackknifing procedure or from residuals of the Procrustean superimposition. In simulation studies where the performance of PACo was compared to another popular global-fit method, ParaFit (Legendre et al., 2002), PACo was superior to ParaFit in its overall performance (Balbuena et al., 2013; Pérez-Escobar et al., 2016). The host phylogeny used in the cophylogenetic analysis was based on the phylogeny by Pisanty et al. (2022) and only *Stylops* species with present hosts in the *Andrena* phylogeny were included. The *Stylops* phylogeny was based on the MCC tree produced by Bayesian inference in this study. Both *Andrena* and *Stylops* trees were pruned with the keep.tip function in package ape v.5.7–1 (Paradis & Schliep, 2019). The cophylogenetic analyses were performed with two randomization algorithms: r0, which conserves the number of interactions of the hosts and with backtracking, which conserves the

interactions of both hosts and parasites. The symmetric argument was set to false, as host evolution tends to drive parasite evolution. For both analyses, the number of permutations was 10,000, and the individual links were assessed with both jackknifing procedure (paco_links) and with residuals (residuals_paco). Smaller residual indicates stronger congruence with a cophylogenetic hypothesis. The contributions of the individual host-parasite links were visualized with cophylo function in package phytools v.1.9–16 (Revell, 2012), and differences in residuals between clades with package ggplot2 v.3.4.2 (Wickham et al., 2016). The statistical significance of the differences between the residuals of clades was assessed with kruskal.test in package stats (R Core Team, 2022).

3. Results

3.1. Phylogenetic analysis and species groups

Out of the 3,913 orthologous genes of the reference dataset, 3,138 were found in the assemblies. Removal of short loci (<100 bp) resulted in a dataset of 2,234 loci which were used with ASTRAL-III (Species Tree, ST), IQ-TREE2 (Maximum Likelihood, ML) and SORTADATE/BEAST (Bayesian Inference, BI). The monophyly of family Stylopidae and the genus *Stylops* was maximally supported by the analyses using outgroups (ST and ML), both recovering *Eurystylops oenipontana* as sister to *Stylops*. However, *Kinzelbachus friesei* together with *E. oenipontana* was recovered as sister to *Stylops* in some of the analyses with third codon

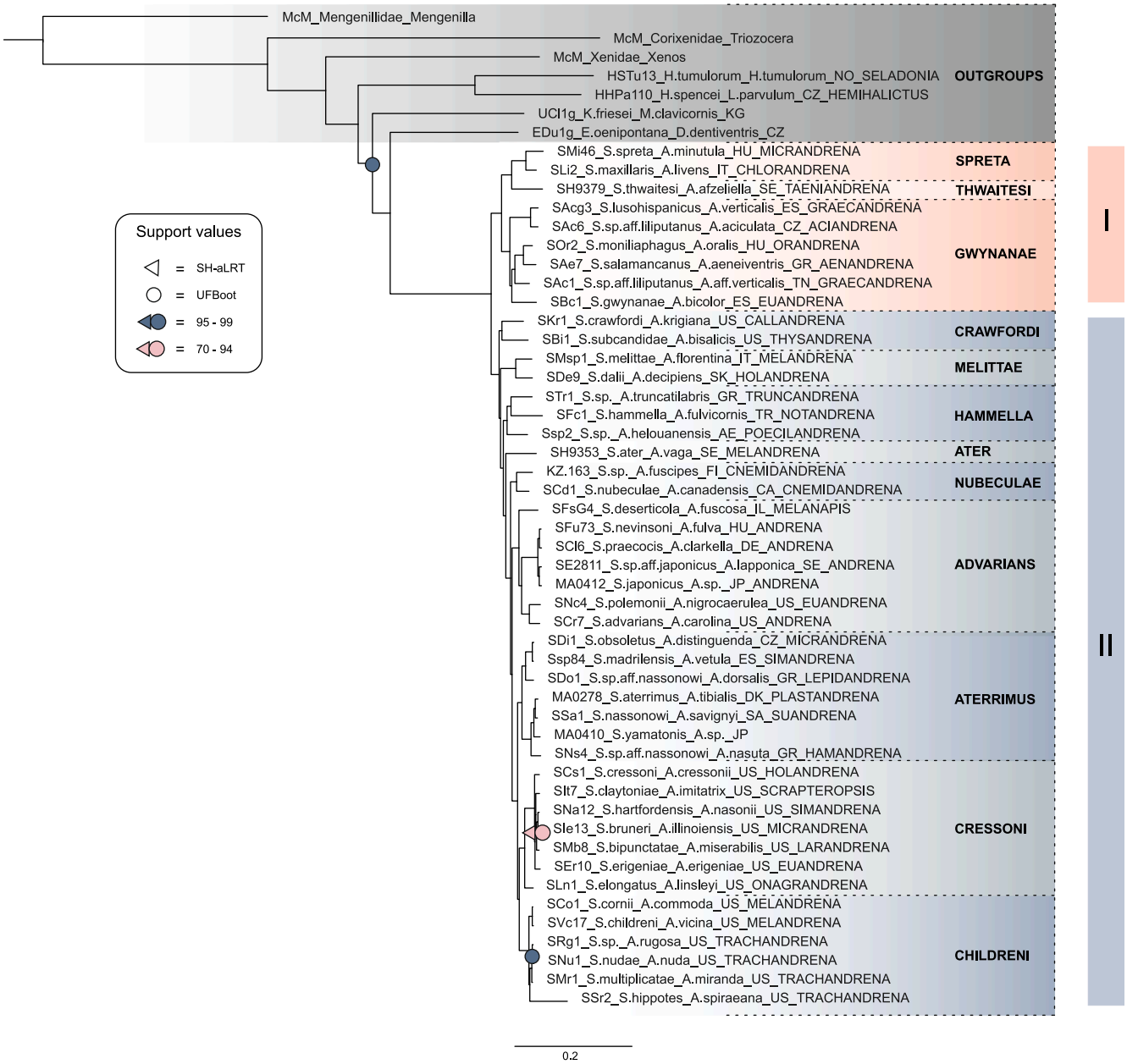

Fig. 2. Phylogeny of *Stylops* based on Maximum likelihood analysis of 2,234 loci in IQ-TREE2. Major clades are denoted by different colours (clade I pink and clade II blue, outgroups in grey). Species groups are delimited by boxes and named after the oldest name in the group. All nodes are maximally supported (100/100) except those indicated with shapes coloured by SH-aLRT (triangle) and UFBoot (circle) values. The scale bar indicates the expected number of substitutions per site. Terminal labels contain the following information separated by underscores: Voucher code, *Stylops* species, Host species (*Andrena*), sampling country in ISO 3166–1 alpha-2 abbreviation code and the host subgenus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

positions excluded (Figs. S6, S9).

All three methods yielded nearly identical topologies for the ingroup with the majority of nodes maximally supported by all metrics (Fig. 2). Two major clades were recovered (I & II), each with multiple smaller clades that we hereafter refer to as 12 named species groups (Fig. 2). Three species groups contained only Nearctic species, seven only Western Palaearctic species and two were geographically mixed. Both the outgroup methods (ST, ML) and the clock model (BI) placed the root at the same position between clade I and II, yielding high confidence in this root position. Topological differences between analyses were restricted to four cases of rearrangements within species groups and only one case including a backbone node between species groups. The latter involved the resolution of the basal node of clade II. ST and one of the BI analyses (TO100) resolved this as a sister group relationship between the *melittae* and *crawfordi* species groups, whereas ML and two of the BI analyses (TO50, NF) resolved this as the *crawfordi* species group being sister to the *melittae* species group + remaining clade II (Fig. 2, Fig. 4, Figs. S3-4, S7, S10-11). In the FcLM topology test a majority of drawn quartets supported a sistergroup relationship between the *melittae* and *crafwordi* groups (80.3 %) (Fig. 3). The topology present in ML and two BI trees (TO50, NF) had much lower support (17.7 %).

Exclusion of short loci from input to ASTRAL-III increased the support values of the nodes with less than maximal support but resulted in an identical topology as with the full dataset (Figs. S3-4). One key node defining a clade including the *advarians*, *aterrimus, cressoni* and *childreni* groups, to the exclusion of the *nubeculae* group, remained poorly supported (0.74) with alternative weakly supported resolutions in some nt12 analyses (Figs. S5-6, S8-9). The exclusion of short loci had ambivalent impact on the support values of nodes in the ML tree and the relative position of *Stylops cressoni* Pierce, 1909 and *Stylops erigeniae* Pierce, 1909 in the *cressoni* species group varied between the two data sets (3,138/2,234 loci, Figs. S7/Fig. 2). The Bayesian analyses showed little sensitivity to missing data in terms of taxon occupancy. Topologies inferred from the different datasets (taxon occupancy 100 %, at least 50 % or no additional filtering for taxon occupancy in the gene selection step) were largely identical. The only disparities were the resolution of the basal node of clade II as mentioned above, the resolution of the *Stylops multiplicatae* Pierce, 1909, *Stylops nudae* Pierce, 1911 and *S. sp.* clade, the position of *Stylops yamatonis* Kifune & Hirashima, 1985 in the *aterrimus* species group and the relative position of *S. cressoni* and *S. erigeniae* in the *cressoni* species group. Most nodes had maximal support by all three datasets (Fig. 4, S10-11), but increasing taxon occupancy also increased overall node support. Rounded to two decimals, the NF tree had five nodes, TO50 three nodes and TO100 only a single node with less than maximal support. BI results of the randomly selected loci for each data set yielded nearly identical topologies, with slight differences in the support values (not shown). Overall, the BI results were topologically more similar to the ML tree (1 or 2 topological differences per tree) than to the ST tree (3 or 4 topological differences per tree). Apart from topological variations already mentioned, the ST tree had a unique variation to the position of *Stylops hippotes* Pierce, 1909 in the *childreni* species group (Fig. S3). This species had an unusually long terminal branch which might affect different types of analyses in disparate ways. The subsequent mentioning of BI results and associated posterior probabilities in the text refer to the TO50 data set, if not stated otherwise. Likewise, references to ML and ST results refer to results from the filtered dataset of 2,234 loci.

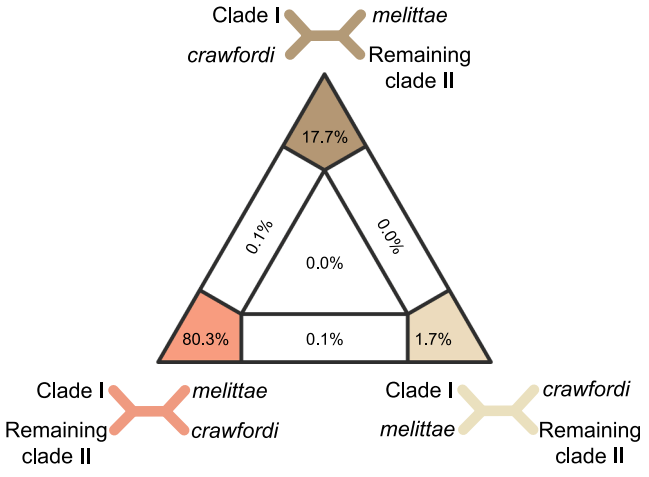

Fig. 3. Four-cluster likelihood mapping results for the resolution of the basal node in clade II. The three alternative resolutions are shown next to the vertices of the triangle. Support for each topology is shown by quartet proportions (in %) mapped on the inside corners of the triangle with corresponding colour.

3.2. Divergence dating and biogeographical history of Stylops

The start of the crown-group diversification of *Stylops* was estimated to be in early Miocene, ca. 22 Ma (95 % HPD: 8.3–35.7 Ma) (Fig. 4). However, the lack of proper calibration points can be seen in the wide confidence interval and the upper and lower limits extend from late Eocene to late Miocene (Fig. S12). Following the split into two main clades in early Miocene the crown ages of clades I and II were both estimated to ca. 17 Ma (95 % HPD: 6.3–28.4/ 6.3–28.3 Ma). The *cressoni* and *childreni* groups had the youngest stem age out of the species groups, ca. 8.4 Ma (95 % HPD: 2.9–13.7 Ma). These groups together with the *advarians* group contained diversifications in the Pleistocene. Model comparisons in BioGeoBEARS supported DEC + J as the best fit model for the biogeographic reconstructions (Table 1). Based on the results, *Stylops* has either Nearctic + Western Palaearctic (47 %, NW) or Western Palaearctic (43,7 %, W) origin but Western Palaearctic + Eastern Palaearctic origin is possible too (WE, 7,4%) (Fig. 4.) The best model without + J was DIVALIKE and with it, *Stylops* was inferred to originate from W (66,6 %) or NW (33,3%) (Fig. S13). In the most likely scenario, there has been four independent dispersal events during Miocene from the Western Palaearctic to the Nearctic region: i) the ancestor of the *crawfordi* group ca. 15 Ma, ii) within the *advarians* group ca. 9 Ma, iii) the ancestor of the clade containing *cressoni* + *childreni* groups ca. 8 Ma. and iv) within the *nubeculae* group ca. 8 Ma.

3.3. Cophylogenetic analysis

The results from global fit analyses in PACo supported congruence between evolutionary histories of *Stylops* and *Andrena* (All $p < 0.001$). This suggests that *Stylops* have not diversified independently of the host phylogeny. At the same time, it is clear from the tanglegram that the explanatory power of coevolution for the diversification of *Stylops* is limited (Fig. 5). Estimation of the individual host-parasite link contributions yielded comparable outcomes across both the jackknifing and residual procedures. The links that contributed most to the signal were the same (Fig. 5). *Stylops* species within clade I had lower residual values on average than species in clade II (Fig. S14, $\chi^2 = 16.755$, df = 1, $p < 0.001$). This finding suggests a more pronounced cophylogenetic signal between species in clade I and their respective hosts in comparison to species in clade II.

4. Discussion

4.1. The phylogeny of Stylops

Despite *Stylops* being the largest genus of the order Strepsiptera, an intrageneric phylogenetic hypothesis has been lacking. Here, we presented the first comprehensive phylogeny for the genus, which included 46 of the estimated 68 species from the Holarctic region. With whole genome sequencing, we were able to acquire a data set of 3,138 loci, yielding a well resolved phylogeny with almost all nodes maximally

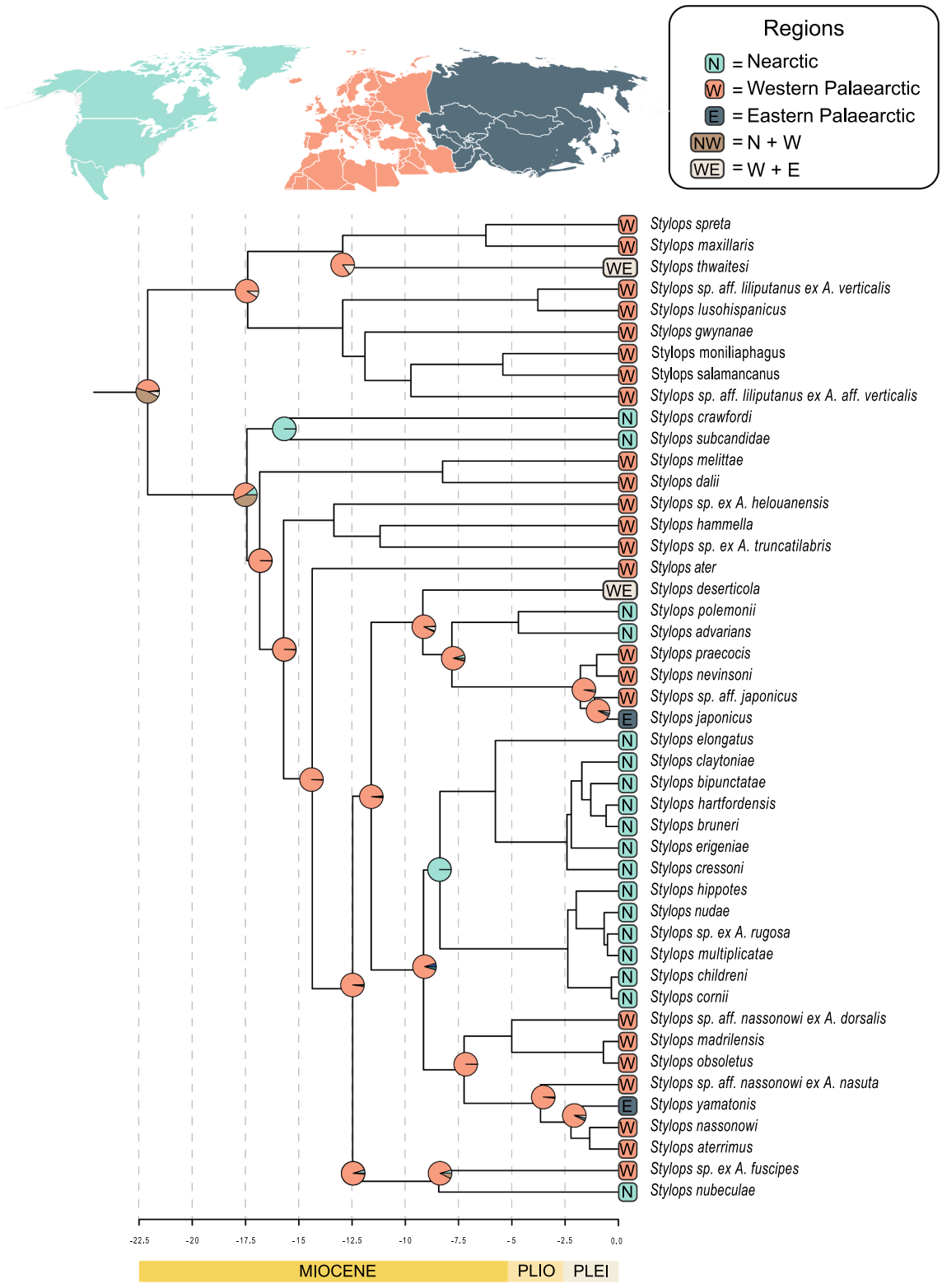

Fig. 4. Dated phylogeny and ancestral-range estimates for *Stylops*. The phylogeny was inferred in BEAST2 with the TO50 data set of 150 genes (inclusion criteria of loci required at least 50 % taxon occupancy). Biogeographical history was inferred for the tree with BioGeoBEARS using DEC + J model. The branch lengths are proportional to divergence times. Circles in nodes include inferred ancestral areas proportional to their likelihood, and colours for each area are given in the legend.

supported. There were only minor discrepancies between topologies from different inference methods, and the root placement was confirmed by two different methods.

Previous higher-level phylogenies have placed *Stylops* as a sister to *Halictoxenos* (Benda et al., 2020; Pohl, 2002; Pohl et al., 2020; Pohl & Beutel, 2005, 2008) or *Kinzelbachus* Özdikmen, 2009 (Kinzelbach, 1990). *Stylops* has also been recovered as sister to *Kinzelbachus* + *Eurystylops* Bohart based on a mitochondrial COI genetree (Jůzová et al., 2015) or hypothesized to be sister lineage of all remaining Stylopidae genera (Bohart, 1941; Ulrich, 1964). *Eurystylops* has been hypothesized

Table 1
Statistical results from BioGeoBEARS including comparison of the fit of different models and model specific estimates for parameters. Included models were Dispersal-Extinction-Cladogenesis (DEC), ML version of Dispersal-Vicariance Analysis (DIVALIKE), Bayesian biogeographical inference model (BAYAREALIKE), each with and without founder-event speciation (+J). LnL = log-likelihood, d = rate of dispersal, e = rate of extinction, j = likelihood of founder-event speciation at cladogenesis, AIC = Akaike's information criterion. Preferred models with and without + J are in bold.

Model	LnL	*d*	*e*	*j*	AIC
DEC	−41.6068417	0.0111346	1.00E-12	0	87.21
DEC + J	−33.3016797	0.00327638	1.00E-12	0.03701827	**72.6**
DIVALIKE	−38.1560412	0.01220782	1.00E-12	0	**80.31**
DIVALIKE + J	−33.3524451	0.00418757	1.00E-12	0.03529246	72.7
BAYAREALIKE	−58.3570811	0.01065938	0.03877957	0	120.7
BAYAREALIKE + J	−34.4924647	0.00292805	1.00E-07	0.03993533	74.98

Fig. 5. A tanglegram of Stylops lineages (right) and Andrena host species (left). Individual host-parasite links and their contribution to cophylogenetic signal are shown with lines, weighted by residuals from PACo. The thicker the line, the stronger the signal. Links of species in clade I are coloured pink, whereas links of clade II are blue. The host phylogeny is based on an Andrena phylogeny by Pisanty et al. (2022) and the Stylops phylogeny is based on the BI tree in this study, both pruned to only include associated species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to be closely related to *Stylops* (Bohart, 1941, 1943), yet it has also been placed as a sister lineage to *Halictoxenos* (Kinzelbach, 1971, 1990). Representatives of *Halictoxenos, Kinzelbachus* and *Eurystylops* were included in our dataset, and most inferences yielded a topology where *Eurystylops* is sister to *Stylops*. This suggests that *Eurystylops* may very well be the sister lineage of *Stylops*. However, as some analyses with third codon positions excluded led to an incongruent, albeit poorly supported resolution where *Eurystylops* + *Kinzelbachus* was sister to *Stylops*, more research is needed to confirm the sister relationship. The relationship to the remaining Stylopidae genera, *Hylecthrus* Pierce, 1909, *Melittostylops* Kinzelbach, 1971, *Crawfordia* Pierce, 1908 and *Rozenia* Straka, Jůzová & Batelka, 2014 was not tested here, but all seem unlikely to be more closely related to *Stylops* based on previous studies (Benda et al., 2020; Jůzová et al., 2015; Pohl et al., 2021; Pohl & Beutel, 2005; Straka et al., 2014).

When the three gene (COI, NADH, EF1) phylogeny of Jůzová et al. (2015) is reinterpreted together with the later assigned species identifications in the preliminary world checklist (Straka et al., 2015), it can be compared to our results. The phylogenies are only partly congruent, which largely can be explained by the non-existent support to the backbone resolution of the 3-gene phylogeny (Jůzová et al., 2015), but also by the root position. In the 3-gene phylogeny *Stylops* is rooted on *Stylops crawfordi* resulting in a highly asymmetric topology. We recover *S. crawfordi* Pierce, 1909 as sister to *Stylops subcandidae* Pierce, 1909 in all analyses, a species in a quite different position in the 3-gene phylogeny. Many species in Jůzová et al. (2015) were in fact only represented by mitochondrial COI including these two species, and when COI was analyzed alone, *Stylops* was instead rooted on *Stylops thwaitesi*

Perkins, 1918. We conclusively infer the root position of *Stylops* between our clades I (*spreta, thwaitesi* and *gwynanae* species groups) and II (remaining species groups). Pruning *S. crawfordi* and *S. subcandidae*, an alternative root placement of the 3-gene phylogeny of Jůzová et al. (2015) will actually recover the same two clades. This shows that it is not just a weakly supported backbone resolution but also root position behind the discrepancies. While we consistently recovered the root between clades I & II, the basal-most node of clade II was the only backbone node showing variation in topology between the different analyses. This node involves the placement of the *S. crawfordi* + *S. subcandidae* clade so it is clear this is among the most difficult lineages to place in the phylogeny, even with thousands of genes.

Several of our species groups were also recovered by Jůzová et al. (2015), including the *melittae-* (*Stylops dalii* Curtis, 1828 and *Stylops melittae* Kirby, 1802), *spreta-* (*Stylops spreta* Perkins, 1918, *Stylops maxillaris* Pasteels, 1949), *aterrimus-*, *cressoni-* and *childreni-* species groups, as well as the isolated position of *S. ater* and partly the *advarians* species group. The Nearctic clade formed by the *cressoni* and *childreni* species groups is sister to the *aterrimus* species group (CCA clade), likewise congruent between the studies. The constituting species of the *hammella* species group is new however, and we recover a different resolution between this, the *advarians* species group, *Stylops ater* and the CCA clade.

The last main difference is the *gwynanae* species group, which is not monophyletic in the 3-gene phylogeny of Jůzová et al. (2015). This might be partly explained by uncertainties in the species diversity and identification of taxa parasitizing hosts from *Aciandrena* and *Graecandrena* (Lähteenaro et al., 2024). Some taxa parasitizing these subgenera could not be assigned to any species (Jůzová et al., 2015; this study) and there seem to be multiple undescribed species involved (Lähteenaro et al., 2024), some of which were excluded from this study. The root position in the 3-gene phylogeny is also likely to explain, at least partly, the dispersal of the species we recover in the monophyletic *gwynanae* species group.

4.2. Phylogenetic relationships of the species groups

Species in large diverse genera can be challenging to grasp, structure and communicate around other than as alphabetical or chronological lists. However, a solid phylogenetic hypothesis opens the possibility of defining informal species groups based on relatedness, facilitating communication and divide-and-conquer stepwise research progress. For practical purposes we therefore defined twelve species groups with main criteria being monophyly under all inference methods. Some groups were limited into one realm, while others were geographically heterogeneous. Likewise, there were species groups which contained several species that parasitized a single *Andrena* subgenus, and species groups where none of the species had shared associations with the same host subgenus.

Clade I consisted of only three Palaearctic species groups, *spreta*, *thwaitesi* and *gwynanae. Stylops thwaitesi* was separated into a monotypic species group since morphologically and based on branch lengths it is quite different from the species in the *spreta* group composed of smaller species. Two species in the *gwynanae* group, *Stylops lusohispanicus* Luna de Carvalho, 1974 and a specimen likely belonging to an undescribed species shared *Graecandrena* as host subgenus but did not have a sister relationship.

The Nearctic *crawfordi* species group, composed of *S. crawfordi* and *S. subcandidae*, was sister to the remaining groups in clade II in the ML and two of the three BI trees. Where this resolution was inferred, it was maximally supported. However, the two species represent a very deep split in the dated phylogeny (Fig. 4), and they parasitize different subgenera of *Andrena*. Likewise, the Western Palaearctic *melittae* species group, composed of *S. melittae* and *S. dalii*, is heterogeneous in terms of parasitized *Andrena* subgenera and was inferred as a sister to the *crawfordi* species group in the ST and one of the three BI analyses. The resolution of this basal trichotomy of clade II was the only backbone node varying between analyses when all codon positions were included. However, based on the performed topology test, the resolution where *crawfordi* and *melitttae* groups are sisters is more likely.

The *hammella* species group included only Western Palaearctic species, *Stylops hammella* Perkins,1918 and two specimens which could not be assigned to any described species. *Stylops ater* seemingly have no close relatives among included species and is therefore delimited as a monotypic species group. *Stylops ater*, sometimes referred to as *Stylops ovinae*, is probably the best studied species of *Stylops* especially in terms of anatomy and development (Fischer et al., 2021; Fraulob et al., 2015; Hoffmann et al., 2023; Jandausch et al., 2022; Löwe et al., 2016; Peinert et al., 2016; Pohl, Gorb, et al., 2020). As the host species *Andrena vaga* Panzer, 1799 form loosely aggregated colonies, the parasite can be more predictably found in numbers compared to many other *Stylops* species whose hosts are solitary. *Stylops ater* has therefore become something of a model species for Strepsiptera.

The *nubeculae* species group was recovered as sister to a larger clade including the *advarians, aterrimus, cressoni* and *childreni*-species groups in most inferences. Only inferences excluding third codon positions resolved the relationship differently, reversing positions of the *nubeculae* and *advarians* groups. The *nubeculae* group contains two species, one undescribed and *Stylops nubeculae* Pierce, 1909, both of which parasitize subgenus *Cnemiandrena* Hedicke,1933. Interestingly, *Stylops* sp. is from the Western Palaearctic region, whereas S. *nubeculae* is Nearctic. The *advarians* group also contained species which parasitize the same subgenus but occur in different realms. Four Palaearctic species had hosts from the nominotypical subgenus *Andrena* Fabricius, 1775 as well as one of the Nearctic species. The clade containing the *advarians, aterrimus, cressoni and childreni* -species groups was fully supported by all metrics in ML and BI trees but had low support values in the ST analysis.

The *cressoni, childreni* and *aterrimus* -species groups formed a strongly supported clade (here referred to as the CCA clade) in all analyses. The Palaearctic *aterrimus* species group was sister to a clade with the Nearctic *childreni* and *cressoni* species groups. The internal nodes of the *aterrimus* species group were maximally supported by all metrics. Two subclades were present: one with Eastern and Western Palaearctic species, and the other one with only Western Palaearctic species.

Nearctic species groups *childreni* and *cressoni* had a sister relationship in the inferred trees. While the *childreni* group contained only species associated with host from subgenus *Melandrena* Pérez, 1890 or *Trachandrena* Robertson, 1902, the *cressoni* group had species with host from seven different subgenera. Both of the groups included closely related species. Early era North American Strepsiptera taxonomist Pierce described *Stylops* species on a single host association principle so that *Stylops* taxonomy followed host taxonomy (Pierce, 1909, 1911, 1918). Later, many of his species were synonymized by Bohart based on their morphological similarities (Bohart, 1936, 1937; Bohart, 1941), while some were categorized to have an uncertain position. Several closely related species in our phylogeny were among the disputed species. For example, Bohart synonymized *Stylops hartfordensis* Pierce, 1909 and *Stylops bruneri* Pierce, 1909 based on the morphology of 1st instar larvae found on the hosts of both species (Bohart, 1941). Later, however, *S. hartfordensis* was elevated again due to the significant differences in both the host subgenera and size of the hosts (Straka et al., 2015). Now that we have sequences from both species, it is evident that Bohart was correct about the affinity of the two species despite hosts differences.

4.3. Age and ancestral ranges of Stylops

Our divergence age estimations mark a first attempt at inferring a temporal window for the diversification of *Stylops*. However, the lack of fossils is a limiting factor and uncertainty intervals are by necessity large. When it comes to age estimates of Strepsiptera, the sister group relationship to Coleoptera has enabled the use of beetle fossils for calibration. Strepsiptera and Coleoptera have been estimated to split ca. 278 Ma (McKenna et al., 2015), ca. 300 Ma (Misof et al., 2014) or ca.

350 Ma (McKenna et al., 2019) and crown age estimates of Strepsiptera range from ca. 108 Ma (Misof et al., 2014) to ca. 230 Ma (McKenna et al., 2019). The oldest known Strepsiptera fossil is †*Cretostylops* Grimaldi & Kathirithamby, 2005 from Cretaceous amber dating to around 99 Ma (Grimaldi et al., 2005). Since by definition Strepsiptera must be as old as Coleoptera and the oldest beetle fossil is almost 300 Ma, there is a huge gap in the early fossil record for Strepsiptera and *Stylops* is a relatively recently diverged lineage.

Given the close evolutionary connection between a parasite and its host, the divergence time of the host becomes a valuable source of information when more precise dating evidence is lacking. In the absence of applicable fossil evidence, we used a secondary host-based calibration. Using host ages as calibration points has been a common practice in groups that do not leave fossils, such as bacteria (Moran et al., 1997). These types of relationship-based calibration approaches are not limited to parasites or pathogens though. The utilization of host-plant divergence times as calibration points has proven to be advantageous in the context of highly specialized plant feeders, such as butterflies (Chazot et al., 2019). Given that *Stylops* is highly specialized into one host genus, it is reasonable to believe that the host age gives a maximal age of the genus. Although it is possible to imagine a scenario in which *Stylops* predates *Andrena* and the current parasitization is a consequence of a host-shift, it is improbable, especially in the light of the evidence for coevolution presented in this study. Given the relatively young age of *Andrena* it is therefore highly likely that also *Stylops* is a young radiation.

This connection between a parasite and its host encompasses spatial events as well. It is important to consider the current and past geographical distributions of the host when examining the distribution patterns of the parasite (Dittmar, 2010). Knowledge on the biogeography of the host helps to understand the biogeographical patterns of the parasite and vice versa (Galbreath & Hoberg, 2011; Šimková et al., 2017). Our results suggest either a Western Palaearctic or Nearctic and Western Palaearctic origin for *Stylops*. Which alternative is favoured over the other depends on the biogeographical model (Fig. 4, S13). A Palaearctic origin of *Andrena* from either Mediterranean region or Central Asia was suggested by Dubitzky et al. (2010), which is congruent with an inferred dispersal event from the Nearctic to the Palaearctic region at the MRCA of *Andrena* (Pisanty et al., 2022). However, Pisanty et al. (2022) and Bossert et al. (2022) inferred a Holarctic origin for *Andrena*. Hence, it appears that while both Holarctic and Palaearctic origins are plausible for *Andrena*, the current evidence supports the former. As the biogeographical inferences of *Andrena* did not include division of the Palaearctic realm to Western and Eastern, *Stylops* ancestral range estimates presented here and those of *Andrena* are not directly comparable. Nevertheless, dispersal events between the Nearctic and Palaearctic realm can be contrasted.

The main clades of *Stylops* (I and II) diversified around 17 Ma. Purely Palaearctic clade I is less diverse than clade II with Palaearctic and Nearctic lineages. The Nearctic lineages resulted from four dispersal events from the Palaearctic to the Nearctic region. Due to the life cycle constraints and biology (e.g. a short-lived free-living male with poor flight capacity), the means of these dispersal events are likely closely tied to the hosts (e.g. Bentz et al., 2006; Moon et al., 2019). The early branching Nearctic *Thysandrena* lineage (Pisanty et al., 2022), could correspond to the oldest Nearctic *Stylops* clade, which contains a species parasitizing bees from subgenus *Thysandrena*. The dispersal event to the Nearctic leading to the *Thysandrena* lineage occurred 14 Ma (HPD 11–17) (Pisanty et al., 2022), while the *crawfordi* group is estimated to have originated approximately 16 Ma (HPD 6–25) in this study. The next two exchange events within lineages parasitizing host from subgenera *Cnemiandrena* and *Andrena* also potentially match dispersal events in the host biogeography around late Miocene–Pliocene (Pisanty et al., 2022). The fourth dispersal event leading to the *cressoni*- and *childreni*-species groups gave rise to most of the Nearctic species included in this study. However, some of those species may be synonyms, as they parasitize hosts from the same subgenus and terminal branch lengths are for some species minute.

As the interchange of *Andrena* between the Nearctic and Palaearctic regions has been suggested to traverse via the Bering land bridge, this is a probable route for *Stylops* as well (Pisanty et al., 2022). The Bering land bridge was present and suitable for exchange of temperate flora and fauna during all of Miocene and into Pliocene (Sanmartín et al., 2001; Wen et al., 2016), fitting our estimated dispersal event ages. However, the support for either Western Palaearctic or Nearctic and Western Palaearctic origin for *Stylops* proposes another possible scenario. Early exchanges between the Nearctic and Western Palaearctic may have traversed the North Atlantic Land Bridges (NALBs). While the timing of the termination of NALBs has been debated over the years (Denk et al., 2011), a growing body of evidence indicates that it served as a possible dispersal route in the early Miocene (eg. Denk et al., 2010; Jiang et al., 2019). The start of crown-group diversification of *Stylops* coincides with a time period, when *trans*-Atlantic distributions were more common than *trans*-Beringian (Sanmartín et al., 2001). This supports the hypothesis of Nearctic and Western Palaearctic origin of *Stylops*. It is important to note, however, that our ancestral range estimation may have been affected by sampling bias towards Western Palaearctic taxa, which might have led to an underestimation of Eastern Palaearctic nodes (Liu et al., 2022).

4.4. Coevolutionary patterns with Andrena

Understanding relationships between species within a genus is particularly pivotal when studying parasites, since parasite relatedness influences host-parasite interactions. Under the assumption that niche preference is a conserved trait (Münkemüller et al., 2015; Peterson, 2011; Peterson et al., 1999; Wiens et al., 2010), close phylogenetic relatedness of parasite species increases the likelihood of them occupying similar ecological niches (Poulin et al., 2011). This in turn may suggest that they are more likely to parasitize host species that are closely related, although it is debatable if the ecological niche should be seen as the host itself, or the ecological niche of the host. The latter would also require that niche preference of the host is a conserved trait.

Congruence between host and parasite phylogenetic relationships was visible in some clades in this study. Species utilizing closely related host species from subgenus *Andrena* were all within the same species group, though there was a division into Palaearctic and Nearctic species. A similar pattern was seen with species parasitizing hosts from subgenus *Cnemiandrena*. Furthermore, we detected a significant evolutionary dependency between *Stylops* and *Andrena*. The dependency was stronger between the less diverse Palaearctic clade I than with the more diverse clade II with Palaearctic and Nearctic lineages. This might be due to species in clade I sharing longer geographical history with their associated hosts than species in clade II. If a parasite is extensively dependent on its host for dispersal over an extended period, the mere presence of these shared biogeographic histories might be enough to explain the congruence observed in phylogenetic relationships (Althoff et al., 2014). *Stylops* are dependent on *Andrena* for long distance dispersal and whereas clade I share its entire history in the Palaearctic with its host species, clade II has multiple dispersal events into the Nearctic. These dispersal events may have created opportunities for adaptive radiation in the form of new host species. Indeed, clade II contains multiple recently diversified lineages, such as the *cressoni*- and *childreni* species groups in the Nearctic. These radiations might result from host-shift speciation, a prevalent driver of parasite diversification (de Vienne et al., 2013), rather than cospeciation. This would weaken the cophylogenetic signal.

Interestingly, the host subgenus, which supposedly reflects close phylogenetic relatedness between the hosts, did not always predict close relatedness of *Stylops* (Table S15). *Stylops* parasitizing hosts from subgenera *Melandrena, Euandrena* and *Micrandrena* were in three different species groups and *Stylops* parasitizing *Simandrena* and *Holandrena* in two different groups. Mining bees of these subgenera are found both in

Western Palaearctic and Nearctic regions (Michener, 2007). In all the cases where one host subgenus was parasitized by members from multiple species groups, associated *Stylops* species came from both Palaearctic and Nearctic regions. For example, *Simandrena* is associated with one Western Palaearctic and one Nearctic *Stylops* group. This suggests that subgenus *Simandrena* was colonized independently by *Stylops* in the two regions at least one of which must have included host shifting. This might have followed from expansion into new regions such as the Nearctic as discussed above. An alternative explanation for distantly related *Stylops* parasitizing bees from same subgenus could be that the host subgenus is polyphyletic. Pisanty et al. (2022) constructed a molecular phylogeny of subfamily *Andreninae*, which included 98 out of 104 *Andrena* subgenera and showed that most of them were either para- or polyphyletic. However, all the subgenera that hosted multiple *Stylops* species groups in this study are monophyletic after the treatments by Pisanty et al. (2022) which we used. It is possible, that the host species not included in the *Andrena* phylogeny remain in a subgenus, which does not reflect relatedness. Either none or only some host species from subgenera *Holandrena*, *Melandrena* and *Micrandrena* were present in the phylogeny. However, all the host species from *Euandrena* and *Simandrena* included in this study, were included in the *Andrena* phylogeny as well. As the monophyly of those is well-supported, even distantly related *Stylops* may have closely related hosts.

Even though the phylogenetic relatedness of host species often influences host-range of a parasite (Krasnov et al., 2004; Mouillot et al., 2006), host niche attributes or attributes of the parasite itself can surpass its impact (Clark & Clegg, 2017; Johnson et al., 2002). Both the flight period and food plant of the *Andrena* bees facilitate infection by *Stylops* due to the manner of dispersal by *Stylops*. The free-living first instar of *Stylops* is the host-seeking life stage of the parasite. The first instars use phoresy to reach a host in its larval stage by hitching a ride from a flower-visiting parent bee to its nest (Cook, 2014; Kathirithamby, 1989). Temporal overlap between the active season of *Andrena* and host-seeking first instar of *Stylops* is needed for this interaction to succeed. Another factor limiting host availability is the food plants of *Andrena* bees, since the first instar can only utilize bees that visit the particular plant. Depending on the conditions, host shifts can occur either between closely related hosts or distantly related hosts (de Vienne et al., 2007). If more distantly related bee species share both the same flight period and food plants, host shifts between *Stylops* that parasitize them are more likely than between more closely related bee species without shared phenology and feeding niche. If the host shift into a distantly related host leads to speciation, closely related parasite species may have distantly related hosts. Since phoresy has been shown to create opportunities for host shifts between distantly related hosts and thus incongruence between host and parasite phylogenies (Johnson et al., 2002), this might be one explanation for the paraphyly of *Stylops* parasitizing bees from the same subgenus.

5. Conclusions

Stylops is the iconic genus upon which Kirby (1813, along with the genus *Xenos*) established the new insect order Strepsiptera. It is also the most diverse of all Strepsiptera genera. Here we have presented the first genomic study into the phylogeny of *Stylops*, providing a phylogenetic basis for classifying species into 12 natural species groups. Using whole genome sequencing, we recovered a well-supported phylogeny of 46 included species and inferred the root between the *spreta* + *thwaitesi* + *gwynanae* species groups and remaining *Stylops*. Multiple inference methods supported this root position, as well as a sister relationship between *Stylops* and *Eurystylops*. However, as an alternative sister relationship between *Stylops* and *Eurystylops* + *Kinzelbachus* was supported in some analyses, the phylogeny of Stylopidae genera needs further study. While Nearctic and Western Palaearctic species were well covered, future studies may benefit from including more Eastern Palaearctic species. Our dating and biogeographical analyses estimate that extant *Stylops* species started diverging in late Oligocene or Miocene with a Western Palaearctic or Western Palaearctic and Nearctic origin. There were four dispersal events from the Palaearctic to Nearctic region during Miocene consistent with favourable conditions for exchange of temperate fauna across Beringia and possibly across a North Atlantic land bridges for the earliest event. Our cophylogenetic assessment using a global-fit method indicated coevolution between *Stylops* and its *Andrena* hosts, although ample host shifting was also apparent. The intertwined evolutionary history of parasites and hosts was reflected in inferred dispersal events for *Stylops* that potentially could match, and depended on, contemporary inferred events in the host's biogeographical history. Yet, the relative importance of cospeciation versus host-shift speciation as drivers of diversification in *Stylops* remains unquantified.

CRediT authorship contribution statement

Meri Lähteenaro: Investigation, Data curation, Formal analysis, Writing – original draft. **Daniel Benda:** Writing – review & editing, Resources, Data curation. **Jakub Straka:** Writing – review & editing, Supervision, Resources, Conceptualization. **Johan A.A. Nylander:** Writing – review & editing, Supervision, Formal analysis. **Johannes Bergsten:** Writing – original draft, Conceptualization, Funding acquisition, Project administration, Supervision.

Data availability

The 3,138 loci dataset that supports the findings of this study and other relevant associated files are openly available in SciLifeLab Data Repository http://doi.org/10.17044/scilifelab.24793227.

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Ethics approval statement.

Ethical approval was not required for the execution of this research owing to the fact that the research organisms comprised of invertebrates not affected by any guidelines.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2024.108068.

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