Mind the Outgroup and Bare Branches in Total-Evidence Dating: a Case Study of Pimpliform Darwin Wasps (Hymenoptera, Ichneumonidae)

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Abstract.—Taxon sampling is a central aspect of phylogenetic study design, but it has received limited attention in the context of total-evidence dating, a widely used dating approach that integrates molecular and morphological information from extant and fossil taxa. Here we assess the impact of commonly employed outgroup sampling schemes and missing morphological data in extant taxa on age estimates in a total-evidence dating analysis under the uniform tree prior. Our study group is Pimpliformes, a highly diverse, rapidly radiating group of parasitoid wasps of the family Ichneumonidae. We analyze a data set comprising 201 extant and 79 fossil taxa, including the oldest fossils of the family from the Early Cretaceous and the first unequivocal representatives of extant subfamilies from the mid-Paleogene. Based on newly compiled molecular data from ten nuclear genes and a morphological matrix that includes 222 characters, we show that age estimates become both older and less precise with the inclusion of more distant and more poorly sampled outgroups. These outgroups not only lack morphological and temporal information but also sit on long terminal branches and considerably increase the evolutionary rate heterogeneity. In addition, we discover an artifact that might be detrimental for total-evidence dating: “bare-branch attraction,” namely high attachment probabilities of certain fossils to terminal branches for which morphological data are missing. Using computer simulations, we confirm the generality of this phenomenon and show that a large phylogenetic distance to any of the extant taxa, rather than just older age, increases the risk of a fossil being misplaced due to bare-branch attraction. After restricting outgroup sampling and adding morphological data for the previously attracting, bare branches, we recover a Jurassic origin for Pimpliformes and Ichneumonidae. This first age estimate for the group not only suggests an older origin than previously thought but also that diversification of the crown group happened well before the Cretaceous–Paleogene boundary. Our case study demonstrates that in order to obtain robust age estimates, total-evidence dating studies need to be based on a thorough and balanced sampling of both extant and fossil taxa, with the aim of minimizing evolutionary rate heterogeneity and missing morphological information. [Bare-branch attraction, ichneumonoids, fossils, morphological matrix, phylogeny, RoguePlots]
Unfortunately, these different implementations of TED have often reported disagreeing age estimates (Grimm et al. 2015; Herrera and Davalos 2016; Harrington and Reeder 2017; Kealy and Beck 2017; Gustafson et al. 2017), revealing that our understanding of the inner workings of this method is still regrettably patchy (Parins-Fukuchi and Brown 2017).

Incorporating more complex models often requires more and better data for improved estimation of model parameters. With the development of next-generation sequencing technologies, acquiring large amounts of molecular data is no longer a problem (Deluc et al. 2005; McCormack et al. 2015; Misol et al. 2014), but taxon sampling is still a major limiting factor in phylogenetic study design. Besides the sampling of the focal, ingroup taxa, the choice of outgroup also requires special attention: it should ideally include a sufficient sample of taxa that are closely related to, but clearly different from the ingroup (Wheeler 1990; Nixon and Carpenter 1993; Giribet and Ribera 1998; Graham et al. 2002; Philippe et al. 2011). A poorly chosen outgroup can significantly affect topology estimates in nonclock analyses by introducing or at least exacerbating long-branch attraction (Graham et al. 2002; Holland et al. 2003; Philippe et al. 2011) and/or by increasing compositional heterogeneity of sequences and among-lineage rate variation (Tarrio et al. 2000; Rota-Stabelli and Telford 2008; Borowiec et al. 2019). Numerous simulations and empirical studies have demonstrated positive effects of improved taxon sampling on the estimation of topology (Graybeal 1998; Dunn et al. 2008; Heath et al. 2008; Klopfstein et al. 2017), branch lengths (Fitch and Bruschi 1987; Pick et al. 2010), and parameters of evolutionary models (Zwickl and Hillis 2002; Heath et al. 2008). Nevertheless, thorough taxon sampling is not always easy to achieve, especially in very species-rich groups.

Several studies have investigated the impact of poor taxon sampling on age estimates in molecular dating analysis and demonstrated its severe negative effect. The effect was most pronounced when taxon sampling strategy led to strongly imbalanced phylogenetic trees and/or high among-lineage rate variation, which could not be adequately accommodated by existing rate smoothing algorithms or relaxed molecular-clock models (Milne 2009; Soares and Schrago 2012, 2015; Wertheim et al. 2012; Duchêne et al. 2015, 2014). Duchêne et al. (2015) have shown that the effect of tree imbalance on age estimates is even larger when heterochronous sequences are included, as is the case in molecular tip-dating with ancient DNA or in virus studies; a similar effect can be expected when fossil taxa of different ages are included as tips. Both the tree imbalance and high among-lineage rate variation can be introduced through a poorly chosen outgroup, as demonstrated for nonclock analyses (see above), but the outgroup choice notably has received little attention in molecular dating analyses, where a molecular-clock model can infer the root of the tree and thus makes the addition of an outgroup unnecessary. Accordingly, the outgroups then can be excluded from dating analysis to decrease the heterogeneity of evolutionary rates across the tree (Welch and Bromham 2005).

In practice, however, most of the recent TED studies included one to a handful of outgroup taxa, which are thus severely underrepresented compared to the ingroup, both in terms of morphological characters and fossils (Ronquist et al. 2012a; Arcila et al. 2015; Dornburg et al. 2015; Close et al. 2016; Lee 2016; Kittel et al. 2016; Herrera and Davalos 2016; Bannikov et al. 2017; Wang et al. 2018; Paterson et al. 2020). The reasons for including these outgroups, despite their potentially negative effects, is seldom reported in these studies and might vary between authors. They might not trust the clock model enough to rely on it for proper rooting, especially when rate heterogeneity is large, which has led to topological artifacts in the past (Ronquist et al. 2012a). Or they might feel uncomfortable with estimating the root age of their ingroup without providing additional taxa that branch off earlier than that node of main interest. The habit might be another incentive to stick to outgroups, which are standard for rooting nonclock trees. And finally, systematists might simply assume that adding more taxa and thus more data to analysis will overall improve the results. We here aim to systematically test the influence of outgroup sampling on age estimates in TED, using parasitoid wasps of the family Ichneumonidae as a case study.

The Ichneumonidae, or Darwin wasps (Klopfstein et al. 2019b), is the most species-rich family of parasitoid wasps, with more than 25,000 described species (Yu et al. 2016), and at the same time one of the most severely understudied taxa. We focus on Pimpliformes, a monophyletic group comprising nine subfamilies: Acaenitinae, Collyriinae, Cylocerinae, Diacritinae, Diplazonitinae, Orthocentrinae, Pimplinae, Poemeniinae, and Rhyssiniae (Wahl and Gauld 1998; Quicke 2014; Klopfstein et al. 2019b). Pimpliformes are especially interesting from a biological perspective since they cover nearly the entire diversity of hosts and parasitoid strategies known from ichneumonoids (Broad et al. 2018). They oviposit into (endoparasitoids) or onto (ectoparasitoids) their host, which they either permanently paralyze (idiobionts) or allow to continue developing (koinobionts). Recorded hosts span almost all holometabolous insect orders, as well as spiders (Araneae). Several attempts have been made in the past to reconstruct the evolution of important biological traits in Pimpliformes (Wahl and Gauld 1998; Gauld et al. 2002; Quicke 2014), but their conclusions were highly dependent on the stability and resolution of the pimpliform phylogeny, which is still in part unresolved (Klopfstein et al. 2019b).

Even less-well understood than the sequence of events during the radiation of Pimpliformes is their timing and thus ecological context. The fossil record of ichneumonoids is very poorly studied, and most described species come from just a handful of localities...
(Menier et al. 2004). It starts in the Early Cretaceous with the extinct subfamily Tanychorinae (Kopylov 2010a), but the affiliation of this subfamily with Ichneumonidae is somewhat unclear, as its wing venation is intermediate between Ichneumonidae and their sister family Braconidae (Sharkey and Wahl 1992). The Palaeoichneumoninae (Kopylov 2009), also extinct and of a similar age, are thus usually referred to as the oldest ichneumonids. All remaining ichneumonid fossils from the Cretaceous period have been classified in the extinct subfamilies Labenopimplinae and Novichneumoninae (Kopylov 2010a; Kopylov et al. 2010; Li et al. 2017), except for a single amber fossil that was tentatively placed in the extant subfamily Labeninae (McCormack et al. 2013). The oldest fossil associated with an extant pimpliform subfamily (and an extant genus) is an acmaetine, Phaenolobus arvenus Piton, from the latest Paleocene. However, its placement is questionable due to poor preservation, and more reliable records come from two Early Eocene localities, the Green River Formation and Messel Pit (Spasojevic et al. 2018a,b).

No studies have to date attempted to infer the age of Ichneumonidae as a whole or of Pimpliformes in particular. The only previous studies with some bearing on the question are either concerned with the sister family Braconidae (Whitfield 2002) or with the entire order Hymenoptera (Peters et al. 2012) using the MAFFT v.7.017 plug-in and the “translation alignment” option was used for protein-coding genes, which for 57 taxa were extracted

in Supplementary File S1 available on Dryad at https://dx.doi.org/10.5061/dryad.m0cfpxnzm.

We aimed to get a good representation of fossil taxa from different time periods: from the oldest ichneumonids from the Early Cretaceous to fossils from the latest Oligocene period (Supplementary File S2 available on Dryad). Due to the controversial position of the extinct subfamily Tanychorinae, which is somewhat intermediate in morphology between Ichneumonidae and Braconidae, and poor sampling of the morphological diversity in Braconidae, we excluded the two Tanychorinae fossils from most analyses. However, we also ran two analyses under different outgroup sampling schemes with Tanychorinae included to assess their placement and the impact of their inclusion on age estimates (see below).

Morphological and Molecular Data

We used the morphological matrix from Klopfstein and Spasojevic (2019), but with a strongly expanded taxon sampling. Numerous additional character states were defined to capture the added specimen diversity. The complete morphological matrix consists of 222 morphological characters coded initially for 150 extant and 79 fossil taxa. After observing attraction of fossil taxa to some nonpimpliform ichneumonids for which we had not yet coded any morphological data (“bare-branch attraction,” see Results section), we added another 20 extant taxa to the morphological matrix, increasing the total number of scored extant taxa to 170 (Supplementary File S3 available on Dryad, also available at MorphoBank http://morphobank.org/permalink/?P3821). Morphology was scored for the same species from which we obtained molecular data, with a few exceptions where we scored a closely related, congeneric species for morphology (Supplementary File S1 available on Dryad).

Our molecular data set includes nine nuclear protein-coding genes, which for 57 taxa were extracted from a previously compiled hybrid-capture data set (Klopfstein et al. 2019). For another 89 taxa, we newly obtain sequences of these genes using standard PCR and Sanger sequencing (primers and protocols in Klopfstein et al. 2019). For all taxa, we added the D2/D3 portion of the nuclear rRNA gene 28S and the mitochondrial cytochrome c oxidase (COI; but see below) by Sanger sequencing. We achieved good coverage for these 11 genes for the 146 taxa, with an average of seven genes successfully sequenced per taxon (Supplementary File S4 available on Dryad). The sequences were edited and aligned in Geneious 7.1.3 (https://www.geneious.com, Kearse et al. 2012) using the MAFFT v.7.017 plug-in and the algorithm “E-INS-I” (Katoh and Standley 2013). The “translation alignment” option was used for protein-coding genes. The complete alignment contained 6213 base pairs (bp) (Supplementary File S5 available on Dryad). The newly generated sequences are included in some morphologically heterogeneous genera. The complete list of extant taxa is given

in Supplementary File S1 available on Dryad
Tracer v1.7 (Rambaut et al. 2018). All phylogenetic likelihoods and of all parameters for all four runs using We also visually inspected the trace plots of the (ESS), and the potential scale reduction factor (PSRF). of split frequencies (ASDSF), effective sample size (MCMC) diagnostics: the average standard deviation assessed using typical Markov Chain Monte Carlo burn-in of 50%, while the convergence of runs was To summarize the result, we used a conservative to 0.05 in order to increase chain swap probabilities. coefficient was decreased from the default value of 0.1 with a sampling frequency of 1000. The heating and nonpimpliform Ichneumonidae), with outgroup taxa (Chalcidoidea, Evanioidea, Braconidae, 2018). The nonclock analysis included the full set of characters where transition only between neighboring states could be assumed (Supplementary File S6 available on Dryad). This model has been identified states could be assumed (Supplementary File S6 available on Dryad). We can thus for instance, resulted in a median age estimate of 229.3 million years old. We ran four independent runs with four Metropolis-coupled chains each for 150 million generations with a sampling frequency of 1000. The heating coefficient was decreased from the default value of 0.1 to 0.05 in order to increase chain swap probabilities. To summarize the result, we used a conservative burn-in of 50%, while the convergence of runs was assessed using typical Markov Chain Monte Carlo (MCMC) diagnostics: the average standard deviation of split frequencies (ASDSF), effective sample size (ESS), and the potential scale reduction factor (PSRF). We also visually inspected the trace plots of the likelihoods and of all parameters for all four runs using Tracer v1.7 (Rambaut et al. 2018). All phylogenetic analyses in this study were carried out using Bayesian inference in MrBayes 3.2.6 (Ronquist et al. 2012b) on the HPC cluster UBELIX of the University of Bern, Switzerland (http://www.id.unibe.ch/hpc). The final data matrices and resulting consensus trees are available at TreeBASE (http://purl.org/phylo/treebase/phylows/ study/TB2S26969). **Nonclock Analysis**

To assess branch length heterogeneity and to examine the power of our combined molecular and morphological data set to resolve ichneumonid relationships, we first ran a nonclock analysis. As the analysis showed convergence issues with parameter estimation for most of the 3rd codon partitions of the nine nuclear protein-coding genes and for the entire COI gene, we excluded those from all further analyses. The final alignment thus contained 10 genes and 4011 bp. We partitioned the data set by gene and codon position and used PartitionFinder 2 (Lanfear et al. 2017) to identify partitions that could be combined (settings: branch lengths = linked, models = all, model_selection = acc, search = rcluster). In addition, we combined all 1st and all 2nd codon position partitions, respectively, that contained fewer than 30 parsimony informative sites, assuming that substitution model parameter estimates would be very poor for those. The preferred evolutionary model for all partitions was GTR+G+I according to PartitionFinder 2. As MrBayes allows model jumping over the entire GTR subspace, the model parameters for the molecular partitions were set as nst = mixed and rates = invgamma, with all substitution model parameters unlinked across partitions. Morphological characters were analyzed under the Mk model (Lewis 2001), accounting for ascertainment bias (“Mkv,” i.e., only variable characters coded), allowing gamma-distributed rate variation across characters, and ordering all the characters where transition only between neighboring states could be assumed (Supplementary File S6 available on Dryad). This model has been identified as the preferred model for the morphological partition in a previous analysis (Klopfstein and Spasojevic 2018). The nonclock analysis included the full set of outgroup taxa (Chalcidoidea, Evanioidea, Braconidae, and nonpimpliform Ichneumonidae), with Casteruption (Evanioidea) chosen as the functional outgroup. We ran four independent runs with four Metropolis-coupled chains each for 150 million generations with a sampling frequency of 1000. The heating coefficient was decreased from the default value of 0.1 to 0.05 in order to increase chain swap probabilities. To summarize the result, we used a conservative burn-in of 50%, while the convergence of runs was assessed using typical Markov Chain Monte Carlo (MCMC) diagnostics: the average standard deviation of split frequencies (ASDSF), effective sample size (ESS), and the potential scale reduction factor (PSRF). We also visually inspected the trace plots of the likelihoods and of all parameters for all four runs using Tracer v1.7 (Rambaut et al. 2018). All phylogenetic analyses in this study were carried out using Bayesian inference in MrBayes 3.2.6 (Ronquist et al. 2012b) on the HPC cluster UBELIX of the University of Bern, Switzerland (http://www.id.unibe.ch/hpc). The final data matrices and resulting consensus trees are available at TreeBASE (http://purl.org/phylo/treebase/phylows/ study/TB2S26969). **TED Analysis**

In addition to the settings above, in the TED analysis, we used the uniform tree prior and a relaxed clock model with independent gamma rates (IGR). To set priors on the relaxed-clock model parameters, we relied on the calculations from Ronquist et al. (2012a), putting an exponential prior on the IGR variance with a rate of 37.12, and a lognormal prior on the clock rate with a mean on the log scale of −7.08069 and standard deviation of 1; the standard deviation was decreased compared to Ronquist et al. (2012) to put increased weight on lower clock rates and thus older age estimates, assuming that our data set contains enough information from the data to correctly estimate the posterior. The prior on the tree age was set to offsetexp(126, 309), with the offset based on the minimum age of the oldest Evanioidea fossil (Deans et al. 2004), while the mean corresponds to the mean age estimate for Hymenoptera from Ronquist et al. (2012a). In the analyses where nonichneumonid outgroups were excluded (see below), we used the minimum age of the oldest unequivocal ichneumonid fossils (Palaeoichneumoninae, 112.6 Ma) as an offset. We set hard bounds using uniform priors on the age of the fossils according to the range of age estimates for the fossil stratum (Barido-Sottani et al. 2019; Püschel et al. 2020). See Supplementary File S2 available on Dryad for the full list of included fossils and their age intervals with corresponding references. To obtain the effective prior implied by our settings, including the clock rate (which is only effective when running with data), we performed an analysis with molecular and morphological data for extant taxa, but without the fossils and thus without temporal information. We ran two independent runs for 100 million generations each and under three different clock rate settings and always obtained a rather flat age distribution for crown-group ichneumonids (Supplementary File S7 available on Dryad). Our preferred setting for the clock rate (lognormal with mean of log values = −7.08069 and standard deviation = 1), for instance, resulted in a median age estimate of 229.3 Ma and a 95% credibility interval (CI) of 51.3–738.6 Ma (Supplementary File S7 available on Dryad). We can thus assume that any more precise age estimates resulting from the analyses with fossils will indeed be informed by the data and not the prior (Parins-Fukuchi and Brown 2017).

It has been shown that relaxed-clock models can lead to topology artifacts, especially close to the root (Ronquist et al. 2012a). We thus set hard constraints on the monophyly of Braconidae, Ichneumonidae, and Ichneumonoidea (Braconidae + Ichneumonidae), each deposited in GenBank under accession numbers MW048210–MW048331, MW056205–MW056314, and MW123099–MW123898.
Table 1. Summary of outgroup sampling strategies.

| Taxon             | Outgroup setting         |
|-------------------|--------------------------|
|                   | “full outgroup”          |
|                   | “Braconidae”             |
|                   | “Braconidae(1)”          |
|                   | “Xoridinae”              |
|                   | “Xorides-only”           |
| Gasteruption (Eva) | x                        |
| Eusepicladionius (Cha) | x                     |
| Aloedes (Bra)     | x                        |
| Aphidius (Bra)    | x                        |
| Cotusia (Bra)     | x                        |
| Duncus (Bra)      | x                        |
| Diascoides (Bra)  | x                        |
| Macrocotus (Bra)  | x                        |
| Homolobus (Bra)   | x                        |
| Tanycherus (Tan)  | x²                       |
| Khurshidius (Tan) | x²                       |
| Xorides (Xor)     | x                        |
| Odontocolon (Xor) | x                        |
| Aplomerus (Xor)   | x                        |
| Ichneumus (Xor)   | x                        |
| other nonpimpliform ichneumonidsb | x | x |

Notes: Abbreviations in brackets stand for higher level classification of the outgroup taxa: Eva = Evanioidea; Cha = Chalcidoidea; Bra = Braconidae; Tan = Tanychorinae; Xor = Xoridinae.

1Included only in the “full outgroup” and “Xoridinae” analyses.

2For a list of all included nonpimpliform ichneumonid taxa, see Supplementary File S1 available on Dryad.

of which is widely accepted as being monophyletic and have been recovered in previous analyses (Sharkey and Wahl 1992; Dowton and Austin 1994; Peters et al. 2017), as well as in our nonclock analysis. To improve convergence on the clock rate and tree length parameters, we increased the probabilities of the respective MCMC moves (MrBayes command blocks are provided as Supplementary File S8 available on Dryad). MCMC convergence proved much more difficult to attain than in the nonclock analysis and was thus deemed satisfactory when the ASDSF value was below 0.03, ESS values of all scalar parameters were above 100, and PSRF values were below 1.01. The ESS values between 50 and 100 and PSRF values above 1.01 were still accepted for the tree length, tree height, and clock rate parameters in some runs, as it was difficult to get convergence on those even after 150 million generations.

TED Outgroup Settings

We tested five different outgroup sampling strategies (Table 1): i) “full outgroup” (with all outgroup taxa as in the nonclock analysis), ii) “Braconidae” (all braconids and non pimpliform Ichneumonidae, but excluding the two nonichneumonoid taxa), iii) “Braconidae (1)” (a single braconid taxon, Homolobus, the only braconid with both molecular and morphological data, and all nonpimpliform Ichneumonidae), iv) “Xoridinae” (only members of Ichneumonidae as outgroups), and v) “Xorides-only” (a single nonpimpliform Ichneumonidae, Xorides, as outgroup to Pimpliformes).

To set the functional outgroup in each of these cases (i.e., nonichneumonoids, braconids, one braconid, Xoridinae, or Xorides), we enforced monophyly of the remaining taxa through a topology constraint. The fossils were in all cases included in the “Ichneumonidae” monophyly constraint, but excluded from any additional topology constraints (see below). Instead, their placement within Ichneumonidae was estimated entirely from the morphological data. In the “Xoridinae” analysis, we applied a partial constraint to enforce the sister relationship of xoridines and the remaining extant ichneumonoids, for which there is strong evidence from previous phylogenetic studies (Klopfstein et al. 2019a), and at the same time to allow fossils to attach freely to any branch of the tree.

To assess potential bias when excluding the extinct Tanychorinae, we also performed the “full outgroup” and the “Xoridinae” analyses with two members of this subfamily included. In the “full outgroup” case, we constrained Tanychorinae within Ichneumonoidea, but applied a partial constraint on Braconidae and Ichneumonidae, which allowed Tanychorinae to attach to any crown or stem branch of the two families. In the “Xoridinae” analysis, Tanychorinae were constrained to be the sister group to the remaining taxa, thus effectively rooting the tree with this extinct subfamily.

Fossil Placement

We assumed that an erroneous placement of the oldest included fossils would have the greatest influence, if any, on age estimates. We thus used “RoguePlots” as described in Klopfstein and Spasojevic (2018) to examine the placement of the Cretaceous fossils on 1000 evenly sampled trees from the four runs in relevant analyses (R package available at https://github.com/seraklop/RoguePlots). In the “Xoridinae” outgroup setting, the Cretaceous impression fossils were predominantly placed on some
terminal branches leading to nonpimpliform taxa without morphological data (see Results section). We thus ran an additional analysis under the “Xoridinae” outgroup sampling scheme with an improved morphological matrix, which now did not contain any outgroup taxa without morphological data.
followed by the “Braconidae (1)” and “Xorides-only” sampling scheme; the latter resulted in the least precise age estimates (Fig. 2, Supplementary File S11 available on Dryad). The inclusion of two representatives of the extinct Tanychorinae in the “full outgroup” and “Xoridinae” analyses also decreased the precision of the age estimates, while the impact on median age estimate was close to zero in the former and the only minor in the latter analysis (Fig. 2).

Due to these large differences in age estimates, we assessed the impact of the outgroup setting on the variance of the clock rate to detect any pronounced rate heterogeneities between outgroups and ingroups. The estimated variance for the relaxed clock (ICER) varied considerably across the different outgroup settings (Fig. 3) and was nearly twice as high in the analyses with distant outgroups (“full outgroup” and “Braconidae”) compared to the analysis with only close outgroups (“Xoridinae” and “Xorides-only”). The exception was when a single braconid was included, in which case the terminal branch leading to the single braconid species also obtained a much higher clock rate than the remaining branches, but this was not yet sufficient to increase the average clock variance very much (results not shown). This finding confirms the pronounced rate variation among outgroups as observed already in the nonclock tree (Fig. 1), where especially some Braconidae species showed very long terminal branches compared to the ingroup.

From here onwards, we suppose that the age estimates were biased when more distant outgroups were included, as these outgroups introduced high rate variation, were poorly sampled, and no fossils were included that might have provided timely information for the outgroup branches to accurately estimate the evolutionary rate in this part of the tree. This notion is
FIGURE 2. Age estimates in million years for Ichneumonidae and Pimpliformes across different analyses. The median and 95% credibility intervals for different outgroup settings are plotted on the y axis. Numbers in brackets indicate the number of included Braconidae species. Dashed horizontal lines indicate boundaries between geological periods. Analyses with Tanychorinae included are denoted by a fossil drawing (modified after Kopylov 2010a). The insect silhouettes denote the estimated time of radiation of the biggest orders of holometabolous insects (according to Tong et al. 2015), which are the main hosts of ichneumonids: Lepidoptera, Diptera, Coleoptera (Permian), and Hymenoptera (Triassic).

FIGURE 3. Variance of the relaxed-clock parameter as estimated under an independent gamma rates model (IGR). Median and 95% credibility intervals are plotted on the y axis across different outgroup settings. Numbers in brackets indicate number of included Braconidae area. Analyses with Tanychorinae included are denoted by a fossil drawing (modified after Kopylov 2010a).

supported by the smaller clock-rate variance and higher consistency in the age estimates when only close and well-sampled outgroups were included (“Xoridinae” outgroup setting). We also consider age estimates in the “Xorides-only” analysis to be biased, given that it resulted in topological artifacts, poor precision of age estimates, and most importantly, numerous fossils were missing their closest extant relatives under this outgroup setting (see next section). Our preferred analysis is thus the “Xoridinae” outgroup sampling scheme.

Placement of Cretaceous Fossils

Placements of most of the Cretaceous compression fossils in our initial analyses with different outgroup settings were rather similar: they clustered predominantly within crown-groups of nonpimpliform ichneumonid subfamilies (Fig. 4b,c, Supplementary File S12 available on Dryad). The highest placement probabilities were centered around extant Banchinae (Banchus and Apophia) and Tersilochinae (in the case of Labenopimplinae and Tryphopimpla xoridoptera) and/or on the branch leading to Orthopelma (especially in Palaeoichneumoninae), with only a few weakly supported placements in other parts of the tree (less than 10% attachment probability). All Labenopimplinae were placed with the highest probability (34–44%, respectively) on the branch leading to Banchus. The overall support for Labenopimplinae belonging to crown group Banchinae was quite high (46–53%), and it was even higher if we also considered stem Banchinae (63–73%). Tryphopimpla xoridoptera, a fossil...
with highly uncertain taxonomic affinities, was mostly associated with *Apophus* (34%), with a total probability of attachment within crown Banchinae of 55%, which increased to 71% when adding stem placements. In contrast, the small undescribed ichneumonid (3311_856b) from Late Cretaceous Yantardakh amber (Rasnitsyn et al. 2016) was attached with very high probability (97%) to the branch leading to the extant Phygadeuontinae genus *Gelis*. Interestingly, most of the tips to which the Cretaceous fossils attached contained no or only sparse morphological information (Fig. 4b,c), leading us to postulate a "bare-branch attraction" phenomenon—a tendency especially for fossils with uncertain affinities to be attracted to branches leading to extant taxa for which no or very sparse morphological data has been obtained.

We thus repeated the analysis with the "Xoridinae" outgroup setting, after completing the scoring of morphological characters for all ichneumonid outgroup taxa, including those on "attracting," bare branches. The placement of the Cretaceous impression fossils then changed considerably and mostly shifted towards the root of the tree compared to the previous analysis (Fig. 4d). Most of the Cretaceous fossils were now...
FIGURE 5. Age estimates in million years for the nodes of interests in the “Xoridinae” outgroup analyses with and without missing morphological data for some outgroup taxa. The median and 95% credibility intervals are plotted on the y axis. Dashed horizontal lines indicate transitions between major geological periods. The insect silhouettes denote the estimated time of radiation of the biggest orders of holometabolous insects (according to Tong et al. 2015), which are the main hosts of ichneumonids: Lepidoptera, Diptera, Coleoptera (Permian) and Hymenoptera (Triassic).* Pimplinae without the tribe Pimplini.

placed on stem branches of both nonpimpliform and pimpliform lineages, with only small placement probabilities for long branches of outgroup taxa (e.g., Orthopelma, Brachycyrtus). All Labenopimplinae now attached with the highest probability to the stem branch of the pimpliform subfamily Diplazontinae (23–39%) and to a nearby long branch leading to the extant tryphonine genera Zagryphus and Thymaris (22–32%). The probabilities of a placement of the Cretaceous impression fossils with crown Banchinae was now close to zero (Fig. 4d, Supplementary File S12 available on Dryad).

Impact of Fossil Placement on Age Estimates

Although the placement of the Cretaceous fossils changed when the improved morphological matrix was employed, the median age estimates remained relatively stable (Fig. 5), but with a consistent improvement in the precision of the age estimates for all but one of the examined nodes. For example, the width of the 95% CI decreased from 53 Myr to 49 Myr for the ancestral node of Pimpliformes and from 63 Myr to 53 Myr for the ancestor of Diplazontinae (Supplementary Files S8 and S11 available on Dryad).

Bare-Branch Attraction in Simulated Data Sets

Our 50 simulated data sets overall behaved similarly to the original data, in that most nodes of the tree of extinct taxa were recovered well, but a few nodes that were subtended by very short branches in the simulation tree were not resolved. Most of the fossils behaved as rogues—taxa with uncertain placement in phylogenetic analyses, as in the original analyses, and including them removed any resolution from the consensus tree. RoguePlots of the fossils showed a heterogeneous picture, with some fossils placed rather firmly, while others shifted around a lot, again reflecting rather well the situation with the empirical data. Analyses of fossil attachment probabilities for the terminal branches leading to extant taxa confirm that bare-branch attraction occurs in simulated data sets as well (Fig. 6). On average, this probability was higher on bare branches than on nonbare ones, although with a lot of variation. The effect was strongest for the one-half of fossils that were more distant from any extant taxon than the others, which were preferentially attaching to bare branches, while the opposite was true for the fossils with rather close ties to at least one extant taxon. Age also seemed to have an impact, but it was far less strong, as was the influence of the number of characters coded for a fossil (Fig. 6).

Age of Pimpliformes

The age estimates resulting from our preferred analysis, with multiple but only closely related and well-sampled outgroup taxa (“Xoridinae,” Table 2 and Fig. 7), and after filling in the attracting bare branches, suggest that Ichneumonidae originated during the Early Jurassic (95% CI spans most of the Jurassic: 154.0–204.7 Ma) and Pimpliformes during the Middle (95% CI spans most of the Jurassic 144.0–193.4 Ma). The start of the radiation of most of the pimpliform subfamilies is estimated as having occurred in the Early Cretaceous.

DISCUSSION

Impact of Outgroup Sampling on Age Estimates

We show here that with the inclusion of more distantly related and/or poorly sampled outgroups, age estimates
FIGURE 6. Bare-branch attraction in simulated data. Boxplots showing the cumulative probability of a branch leading to an extant taxon to attract a fossil, depending on whether it was bare in the simulation replicate (i.e., it was lacking morphological data) or not. The branches for which morphological data was deleted were chosen randomly for each simulation replicate. The graphs to the right show the same probability, but for subsets of the fossils: the half of the fossils that are more distant versus closer to extant taxa; Cretaceous versus Cenozoic fossils; and finally fossils with fewer than 20 characters coded plotted separately. Distance from any extant taxa thus appears as the most decisive factor here, but beware that we only obtained simulation replicates on a single tree and thus the factors concerning fossil placement are not independent.

TABLE 2. Age estimates from the preferred analysis with credibility intervals for crown group Ichneumonidae, Pimpliformes, and the pimpliform subfamilies.

| Taxon group          | Median (millions of years) | Mean (millions of years) | 95% credibility interval (millions of years) |
|----------------------|-----------------------------|--------------------------|---------------------------------------------|
| Ichneumonidae        | 181.2                       | 181.4                    | 154.0–204.7                                 |
| Pimpliformes         | 167.8                       | 168.7                    | 144.0–193.4                                 |
| Parasitoids of Diptera | 126.2                      | 127.8                    | 107.3–150.0                                 |
| Acerinae             | 127.6                       | 129.0                    | 105.1–156.7                                 |
| Diplozontinae        | 123.5                       | 124.8                    | 98.5–152.0                                  |
| Pseudoemii           | 82.7                        | 83.5                     | 59.1–113.4                                  |
| Rhyssinae            | 96.8                        | 97.7                     | 69.6–129.9                                  |
| Delemosertini        | 79.7                        | 80.6                     | 51.1–111.8                                  |
| Ephialtini           | 103.7                       | 105.4                    | 82.9–128.9                                  |
| Pimplini             | 146.7                       | 147.4                    | 122.1–175.1                                 |
| Theroniini           | 80.4                        | 80.8                     | 54.3–106.7                                  |

Notes: As the subfamily Pimplinae was not recovered as monophyletic, age estimates for the tribes are given (Delemosertini, Ephialtini, Pimplini, and Theroniini). The parasitoids of Diptera here comprise three subfamilies: Cyllocorinae, Diacritinae, and Ortochocorinae (excluding Diplozontinae and the diacritine Ortholaba, which did not form a monophyletic group with them, note that Diacritinae are included in this clade, but there are no host records for this small subfamily).

For pimpliform parasitoid wasps become older and often less precise. Besides creating a highly unbalanced tree in terms of sampling of both extant and fossil taxa, including distant outgroups in our case introduced large variation in the clock rate, which is most likely the main reason for the observed bias in age estimates (Magall et al. 2013; Magallón 2014; Beck and Lee 2014; Beaulieu et al. 2015; King et al. 2017a). In addition, in the absence of time information in the form of fossils along the branches of distant outgroups together, the age estimates might have been driven by our clock rate prior, resulting in older estimates for crown group Ichneumonidae and Pimpliformes in those analyses (see analysis of effective prior in Materials and Methods). Multiple studies have shown that outgroup choice can greatly affect tree topology estimates, especially in cases with a large heterogeneity of branch lengths and uneven taxon sampling (Puslednik and Serb 2008; Ware et al. 2008; Hayes et al. 2009; Thomas et al. 2013; Burleigh et al. 2014; Wilberg 2015). However, the influence of outgroup choice on divergence time estimates, especially in the context of total-evidence dating, has scarcely been studied, and only indirectly (Linder et al. 2005; Soares and Schrago 2012; Duchêne et al. 2015; Matschiner 2019).

In the last 8 years since total-evidence dating became established, most of the studies have included outgroups in their taxon sampling (Ronquist et al. 2012a; Arcila et al. 2015; Dornburg et al. 2015; Close et al. 2016; Herrera and Davalos 2016; Kittel et al. 2016; Lee 2016; Bannikov et al. 2017). We here covered most of the outgroup-sampling schemes found in these studies, from including a single closely related taxon (“Xorides-only” outgroup setting), over a few relatively closely related taxa (“Braconidae” and “Braconidae (1)”), to the inclusion of a series of more to less closely related outgroup taxa (“Braconidae” and “Braconidae (1)”), to the inclusion of a series of more to less closely related outgroup taxa (“full outgroup”). As in most previous studies, our nonichneumonid outgroups were not only sparsely sampled, but were also missing fossils and morphological data for most of the taxa. All these outgroup settings recovered older and usually less precise age estimates than when we restricted our data set to more closely related outgroup taxa, for which greater and more even effort in sampling both extant and fossil taxa had been applied (“Xoridinae” outgroup.
setting). Interestingly, when two potentially transitional fossils between Braconidae and Ichneumonidae (the Tanychorinae) were included, the median age estimates and clock variance were only slightly affected, but their precision dropped significantly. This could result from the rather large uncertainty in the age of these fossils themselves, which results from a disagreement over the age of the Khasurty Formation (Kopylov and Rasnitsyn 2017; PaleoBioDB 2020), and the fact that only very few morphological characters were scored for them (9% and 17%, respectively).

Our results might suggest that many of the previous TED analyses would recover younger and likely more accurate age estimates without outgroups or with either more detailed or more restricted, but more even outgroup sampling. Presumably, the effect would be most pronounced for data sets where there are long, unbroken outgroup branches which introduce large rate heterogeneity across the tree. A similar effect has been demonstrated for node dating in the simulation study by Soares and Schrago (2015), where age estimates were significantly biased when there was a combination of large among-lineage rate variation and poor taxon sampling. As in our case, both accuracy and precision were affected in their simulations: the mean age of the node in question was constantly overestimated and precision severely decreased. Some TED studies already conjectured that high evolutionary rate heterogeneity can lead to biased age estimates (Beck and Lee 2014; Lee 2016; King et al. 2017b; Bagley et al. 2018; Luo et al. 2020). Among-lineage rate heterogeneity can already be identified on a nonclock tree by comparing...
root-to-tip distances of extant taxa and problematic taxa can accordingly be excluded, as we demonstrated here; however, this has rarely been employed in TED studies (but see Grimm et al. 2015).

In addition, more realistic models of morphological evolution might eventually improve the clock-likeness of morphological data sets by improving their match to the underlying evolutionary mechanisms. Some of the aspects for which attempts have already been made to increase realism are asymmetry in transition rates among character states (Lewis 2001; Wright et al. 2016; Pyron 2017), nonstationarity of the evolutionary process (Klopfstein et al. 2015) or even the modeling of undetected character states through hidden Markov models (Tarasov 2019). These models have not been routinely used in TED studies and, at least at present, many of them are either restricted to specific character types or are computationally challenging for large morphological data sets. The latter was also true for our data set when we tried to account for asymmetric transition rates among character states (Lewis 2001; Wright et al. 2016).

Finally, incompletely sampled phylogenies, with respect to fossil and/or extant taxa, could potentially be modeled more adequately with the fossilized birth–death tree prior (FBD) instead of the uniform tree prior used here, but only when model assumptions are satisfied (Zwickl and Hillis 2002). When model assumptions are violated, such as assumptions related to the sampling density of fossil and/or extant taxa, age estimates are quickly compromised (Matschiner 2019; Luo et al. 2020; Püschel et al. 2020). This makes it difficult to apply the FBD prior to the ichneumonid data set at present, as species richness through time even today is still poorly known for this group.

**Bare-branch attraction hampering correct fossil placement in TED.**

In TED analyses, the placement of fossils is solely dependent on the available morphological information for both fossil and extant taxa. The quality of fossil placement based on morphological matrices is thus primarily limited by imperfect preservation of fossils, but also by high levels of morphological homoplasy, which has been reported for ichneumonids (Gauld and Mound 1982; Klopfstein and Spasojevic 2019). We here identified another potentially major issue in TED studies, which we called “bare-branch attraction”: the tendency of fossils which are only distantly related to any of the included extant taxa to attach to terminal branches of extant taxa for which no morphological data have been collected. This artifact exposes the dangers of insufficient sampling of extant taxa for morphology, which can distort fossil placement and consequently age estimates in TED analyses.

Many of the previous TED studies included from a few to more than half of the extant taxa without morphological data or at least with high amounts of missing data (Ronquist et al. 2012b; Arcila et al. 2015; Dornburg et al. 2015; Harrington and Reeder 2017). Guillerme and Cooper (2016) addressed this issue in the context of topology reconstruction in TED analyses. In their simulations, topology estimates were more negatively affected by a large percentage of extant taxa with missing morphological data than by any other analyzed parameter. It remains unclear to what extent their results were influenced by bare-branch attraction, as they did not analyze individual fossil placements, but it is likely that the artifact played a role under their scenario as well.

In our study, the bare-branch attraction was most obvious in the compression fossils from the Cretaceous. With the exception of *T. xoridoptera*, these fossils are all classified in two extinct subfamilies of fossil Palaeoichneumoninae and Labenopimplinae. The phylogenetic position of these subfamilies is unclear, but two options have been suggested: a transitional position between Tanychorinae and extant Ichneumonidae, which would mean they represent stem ichneumonids, or some rather basal position as crown ichneumonids (Kopylov 2009, 2010b). In fact, the name “Labenopimplinae” reflects their similarity to the extant subfamilies Labeninae and Pimplinae (Kopylov 2010b).

We showed that the predominant placement in our initial analysis, of most Labenopimplinae and Paleoichneumoninae with crown group Banchinae and Tersiolinae, was a bare-branch attraction artifact. Among the branches where these fossils attached on the tree, only a single tip (*Apophua*) contained morphological data, while the remaining taxa were only sampled for molecular characters. Some other, younger fossils with rather labile placement also often attached to these branches, which might suggest that when morphological information for the placement of a fossil is limited, TED analyses tend to place a fossil on “bare branches,” especially if those branches are long. Filling in the missing information among extant outgroup taxa was enough to reverse the initially erroneous placements of the Cretaceous fossils. They then instead ended up on rather basal branches of crown group ichneumonids, in accordance with one of the hypotheses suggested at the time of their original description (Kopylov 2009, 2010b).

**Simulations confirm the bare-branch attraction phenomenon.**

Our simulations have confirmed bare-branch attraction as a general phenomenon, which could be reproduced with simulated molecular and morphological data. The distance in branch lengths between a fossil and its closest extant relative emerged as the strongest factor determining the strength of the artifact, along with the fossil age and number of morphological characters coded. This makes intuitive sense: fossils that are close to an extant taxon will on average have high information content in their morphological data, even if it is incomplete, while
fossils that are distant to any extant taxon are notoriously difficult to place and might thus be more prone to falling victim to the bare-branch attraction.

More extensive simulations are needed to further assess the types of biases arising from the bare-branch attraction in recent simulation studies concerning the FBD tree prior, Luo et al. (2020) reported difficulties in recovering correct fossil placement when rate variation across branches was high and when morphological data were not included. This might indicate the bare-branch attraction artifact to be more pronounced how detrimental this artifact is in the contexts of FBD prior. In any case, this phenomenon has the potential to mislead TED analyses and should be accounted for in future studies. Fortunately, the remedy is very simple: morphological data sets of extant taxa should be as complete as possible.

The age of Pimpliformes and the biological context of their diversification.—

Our most credible analysis estimated the median age of the family Ichneumonidae at 181 Ma (95% CI 154.0–204.7 Ma) and of Pimpliformes to 168 Ma (95% CI 144.0–193.4 Ma), with 95% credibility intervals in both cases spanning most of the Jurassic. The estimated age of Ichneumonidae is thus 60–70 Ma older than the oldest certain ichneumonid fossils, which implies a rather long ghost lineage. However, a similar gap exists between the oldest and the second oldest ichneumonid fossils, which is between 26 and 53 Ma, depending on the age of the geological formations in question (Kopylov and Rasnitsyn 2017; PaleoBioDB 2020). This suggests that the ghost range implied by our analysis is not that long after all. Such a gap is even more acceptable if we consider the paucity of Jurassic Hymenoptera fossils in general (Rasnitsyn and Quicke 2002). Furthermore, the last few years have seen unexpected discoveries of fossils that have closed large gaps between much older (molecular) age estimates and the previously known fossil record, for instance in Lepidoptera (Elidk et al. 2018).

Further insights into the age of ichneumonids come from age estimates for their hosts (nearly exclusively holometabolous insects), which had to originate before their parasitoids could radiate. Initially, the radiations of the most species-rich orders of holometabolous insects, Hymenoptera, Coleoptera, Diptera, and Lepidoptera, were believed to have been associated with the radiation of flowering plants and were dated to the Early Cretaceous (Grimaldi 1999; Misof et al. 2014). However, these estimates were later deemed too young, and a Late Permian origin was suggested based on a reanalysis of a large phylogenomic data set (Misof et al. 2014) with more appropriate calibration points (Tong et al. 2015). This later study implies that the major host groups for ichneumonids were already present during the Jurassic, when both Ichneumonidae and Pimpliformes originated, suggesting that the radiation of these parasitoids might have happened only shortly after the radiation of their host groups.

CONCLUSIONS

We have demonstrated that poor outgroup sampling can negatively affect both accuracy and precision of age estimates in total-evidence dating analyses. Even though the exact mechanisms leading to this decrease in performance with the inclusion of increasingly more distant outgroups remain somewhat unclear, the introduction of large among-lineage rate variation and tree imbalance probably play major roles. Thus, to achieve more reliable age estimates, one should consider excluding outgroups altogether from dating analyses, unless they can be sampled adequately. Correct positioning of the root then can be through topology constraints instead, that are based on prior knowledge, or on a nonclock analysis of the data. In case an outgroup needs to be included in the analysis, for whatever reasons, one should sufficiently sample not only molecular but also morphological and fossil data.

We also illustrated the importance of careful consideration of fossil placement in total-evidence dating analyses in order to identify artifacts or biases. The bare-branch attraction artifact that we have discovered here is likely universally problematic for TED. Most concerning is that the artifact most severely affects fossils distant from extant taxa in a phylogeny, such as older and stem-lineage fossils, whose correct placement has proven crucial for reliable time-calibration of phylogenies. Thus, the bare-branch attraction artifact deserves further assessment in the future, through more detailed simulation studies. Nevertheless, it can easily be circumvented by a more complete sampling of morphological data for extant taxa.

Finally, we provided the first age estimate for the extremely diverse group of ichneumonid parasitoid wasps. The Jurassic origin for the family and for Pimpliformes agrees with the timing of the radiation of their major host groups. It remains to be seen how the age estimate for the family will change when more taxa and especially more fossils are included in a TED analysis. As new fossil ichneumonids are being described at a regular pace (Khalaim 2008; Kopylov 2009, 2010b; McKellar et al. 2013; Antropov et al. 2014; Kopylov et al. 2018; Spasojevic et al. 2018a,b), the coming years will certainly provide further evidence for an older age of many groups within this species-rich family. Our insights on taxon sampling will help provide guidance in the design increasingly efficient dating studies, not only in Darwin wasps, but across the entire tree of life.

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Supplementary Material

Data available from the Dryad Digital Repository: https://dx.doi.org/10.5061/dryad.m0cf keypoints.

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