Incompatible Ages for Clearwing Butterflies Based on Alternative Secondary Calibrations

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Abstract.—The recent publication of a time-tree for the plant family Solanaceae (nightshades) provides the opportunity to use independent calibrations to test divergence times previously inferred for the diverse Neotropical butterfly tribe Ithomiini. Ithomiini includes clades that are obligate herbivores of Solanaceae, with some genera feeding on only one genus. We used 8 calibrations extracted from the plant tree in a new relaxed molecular-clock analysis to produce an alternative temporal framework for the diversification of ithomiines. We compared the resulting age estimates to: (i) a time-tree obtained using 7 secondary calibrations from the Nymphalidae tree of Wahlberg et al. (2009), and (ii) Wahlberg et al.'s (2009) original age estimates for the same clades. We found that Bayesian clock estimates were rather sensitive to a variety of analytical parameters, including taxon sampling. Regardless of this sensitivity however, ithomiine divergence times calibrated with the ages of nightshades were always on average half the age of previous estimates. Younger dates for ithomiine clades appear to fit better with factors long suggested to have promoted diversification of the group such as the uplifting of the Andes, in the case of montane genera. Alternatively, if ithomiines are as old as previous estimates suggest, the recent ages inferred for the diversification of Solanaceae seem likely to be seriously underestimated. Our study exemplifies the difficulty of testing hypotheses of divergence times and of choosing between alternative dating scenarios, and shows that age estimates based on seemingly plausible calibrations may be grossly incongruent. [asynchronous association; crown ages; Danainae; dating; herbivory; hostplant; Ithomiini; molecular clock; Nymphalidae; secondary calibrations; Solanaceae.]

Time-calibrated phylogenetic trees have become increasingly attractive to evolutionary biologists because they offer the opportunity to test, albeit indirectly, different aspects of the evolution of a group, including estimating the origin of species interactions (Percy et al. 2004; Wahlberg et al. 2013) and the temporal patterns of clade diversification (Brower 1996; Wahlberg et al. 2009). They are also particularly valuable at discriminating among competing biogeographical hypotheses (e.g., Trewick 2000; de Queiroz 2005; Swenson et al. 2012; Garzón-Orduña et al. 2014; Murienne et al. 2014; Tripp and McDade 2014). Enthusiasm about molecular clock-based hypotheses of diversification has surged with the rise of "relaxed clock models" that integrate evidence from multiple calibration points with inferred rates of DNA sequence divergence, while allowing estimated rates of evolution to vary across the tree (Drummond and Rambaut 2007). Regardless of methodological sophistication, however, dating trees is fundamentally limited by the availability of independent data to calibrate DNA substitution rates. Strictly speaking, accurately dated fossils constitute the only truly "primary" calibration points—age estimates based on independent evidence of the history of the taxon itself, but there are nevertheless a number of sources of error associated with their use (Parham et al. 2012), including potential difficulty in observing morphological synapomorphies and the limitation that a fossil provides only a minimum age estimate for its clade. Another, slightly less direct source of primary calibration

is biogeographical events (Knowlton and Weigt 1998; Percy et al. 2004; Bonacum et al. 2005; Nazari et al. 2007), but the reliability of such points also requires critical assessment (Kodandaramaiah 2011; Heads 2012), and their use may be circular if the purpose of dating is to test biogeographic hypotheses. Also, if tight coevolutionary associations exist between taxa, such as parasites and hosts, plausible primary calibration points for one taxon may reasonably be applied to the other (Farenholz' Rule, Hennig 1966), conceptually mirroring the vicariance of taxa on areas.

For taxa without readily preserved skeletal remains, the poor condition of fossils can complicate the identification of diagnostic features, meaning that such fossils can only be tentatively placed as the sister group to a particular extant taxon. This has been the case for butterflies (de Jong 2007), whose fossil record is not only sparse, but the fossils that exist are often difficult to assign to a particular node on a cladogram. Uncertainty surrounding the ages of various lineages has provoked considerable debate within the field (de Jong 2007; Brower and Vane-Wright 2011). The ultimate limitation, however, is that for some clades there are no known fossils at all. In such cases, researchers may date trees using "secondary" calibrations, which are estimates of divergence times extracted from inferred relaxed clock rates generated by previous analyses that used primary calibrations outside the clade of interest (Wheat and Wahlberg 2013). Unlike primary calibrations, the term "secondary calibrations" (sometimes also called

indirect calibrations) is used more broadly (Forest 2009) to refer to node age estimates obtained from other molecular dating studies that may or may not have included the group of interest, and that are used to interpolate or extrapolate divergence times of descendent or related clades. In this study we follow these respective definitions of primary and secondary calibrations. Although not ideal, the use of secondary calibrations has become commonplace in the literature (Müller et al. 2010; Heikkilä et al. 2011; Price et al. 2011; Condamine et al. 2012; Müller et al. 2013; Matos-Maravi et al. 2013; more references below), and has been critically discussed only rarely (Graur and Martin 2004; Sauquet et al. 2012; Wahlberg and Wheat 2013).

The largest time-tree for butterflies to date is that of Wahlberg et al. (2009, hereafter "W09") for 399 genera of Nymphalidae, a large and charismatic family of butterflies that has been the subject of much historical and recent research. W09 not only had the largest sample of butterfly taxa and molecular data (combined with morphological data) at the time, but also was the first to use a relaxed molecular clock, calibrated with both fossils and hostplant ages, to provide a baseline of divergence times for the entire family. Since then, this dated tree has served as a working hypothesis and as source of secondary calibrations for numerous other studies (Kodandaramaiah et al. 2010; Müller and Beheregaray 2010; Simonsen et al. 2010; Wahlberg and Rubinoff 2011; Mullen et al. 2011; Penz et al. 2011, 2012; van Velzen et al. 2013), including studies of Ithomiini (Elias et al. 2009), a neotropical tribe of Nymphalidae (cf. Brower et al. 2006) referred to colloquially as "clearwing" butterflies due to the transparent wings of many species.

Ithomiini includes approximately 370 species (Lamas 2004) grouped into 10 subtribes (Brower et al. 2014, hereafter "B14"), and exhibits an impressive diversity of aposematic wing color patterns. These butterflies are distributed throughout the submontane and lowland forests of Central and South America, where they are involved in Müllerian mimicry complexes with one another and with species in Heliconiinae, Danaini, and several other butterfly and moth groups (Bates 1862; Beccaloni 1997). The group is increasingly well-studied from ecological, evolutionary and biogeographical perspectives (Willmott and Mallet 2004; Willmott and Freitas 2006; Elias et al. 2009; Hill et al. 2012; Garzón-Orduña et al. 2014), and represents an important exemplar in the narrative of Amazonian diversification.

Most ithomiines are obligate herbivores of plants in Solanaceae. A single origin of this host association is implied (B14), and some genera of Ithomiini are restricted to feeding oligotrophically on a single genus or clade of nightshades (Brown 1987; Willmott and Freitas 2006). The diversity of Ithomiini has been attributed to their use of Solanaceae as larval hostplants (Brown 1987; Willmott and Freitas 2006), with a shift from Apocynaceae (the putatively ancestral Ithomiini hostplant family shared with sister groups

Danaini and Tellervini; Ackery 1988) to Solanaceae feeding apparently resulting in an accelerated rate of diversification in some clades of Ithomiini (Fordyce 2010; Peña and Espeland 2015).

The recent publication of a comprehensive time-tree for Solanaceae (Fig. 1, modified from Särkinen et al. 2013; hereafter "S13"), including all solanaceous ithomiine larval hostplant genera, allows us to calibrate the B14 ithomiine tree with ages of clades of Solanaceae and to test divergence times for Ithomiini inferred by W09. Furthermore by using W09's divergence times to calibrate B14, we can also compare results from two different sets of secondary calibrations. This is important for two reasons: first, as mentioned above, numerous butterfly researchers have extracted the ages of clades of interest from W09 for secondary calibrations in their own studies. The reliability of such calibrations is critical to the precision and accuracy of subsequently extrapolated temporal inferences. Second, Sauquet et al. (2012) have discussed the challenges of using secondary calibrations versus fossils, but if the only option is to use secondary calibrations, is there a reliable or defensible way to choose one set of calibration points over another? Because of the higher-level scope of W09's study, secondary calibrations from that data set could provide a more comprehensive picture of rate heterogeneity among nymphalid lineages and therefore might be hypothesized to reflect the history of the sampled taxa more accurately. Alternatively, dates extracted from Solanaceae could be viewed as the closest approximations to "real" extrinsic calibrations for Ithomiini, and as such, truly independent data. The empirical consequences of using two independently derived sets of secondary calibrations have not yet been fully explored in the literature.

Here we present novel time-calibrations for Ithomiini based on patterns of hostplant use. We then: (i) compare hostplant-based divergence times to divergence times published by W09 for comparable nodes of Ithomiini; (ii) use W09's ithomiine divergence time estimates as secondary calibration points to generate a new time-tree for our more taxonomically diverse ithomiine data set and compare it to the hostplant-based timetree. We also explore the effects of several other analytical parameters on clade age estimation, such as number of taxa sampled, amount of sequence data, and alternative placements of calibrations. Finally, we discuss how the different estimates of divergence times correlate with patterns of ithomiine natural history and biogeography.

METHODS

Our analyses of divergence times were conducted in BEAST 2.1.3 (Bouckaert et al. 2014) and used two sets of secondary calibrations: the first set corresponds to divergence times for selected clades of Solanaceae (hereafter "hostplant calibrations") extracted from S13's timetree, and the second set comes from divergence
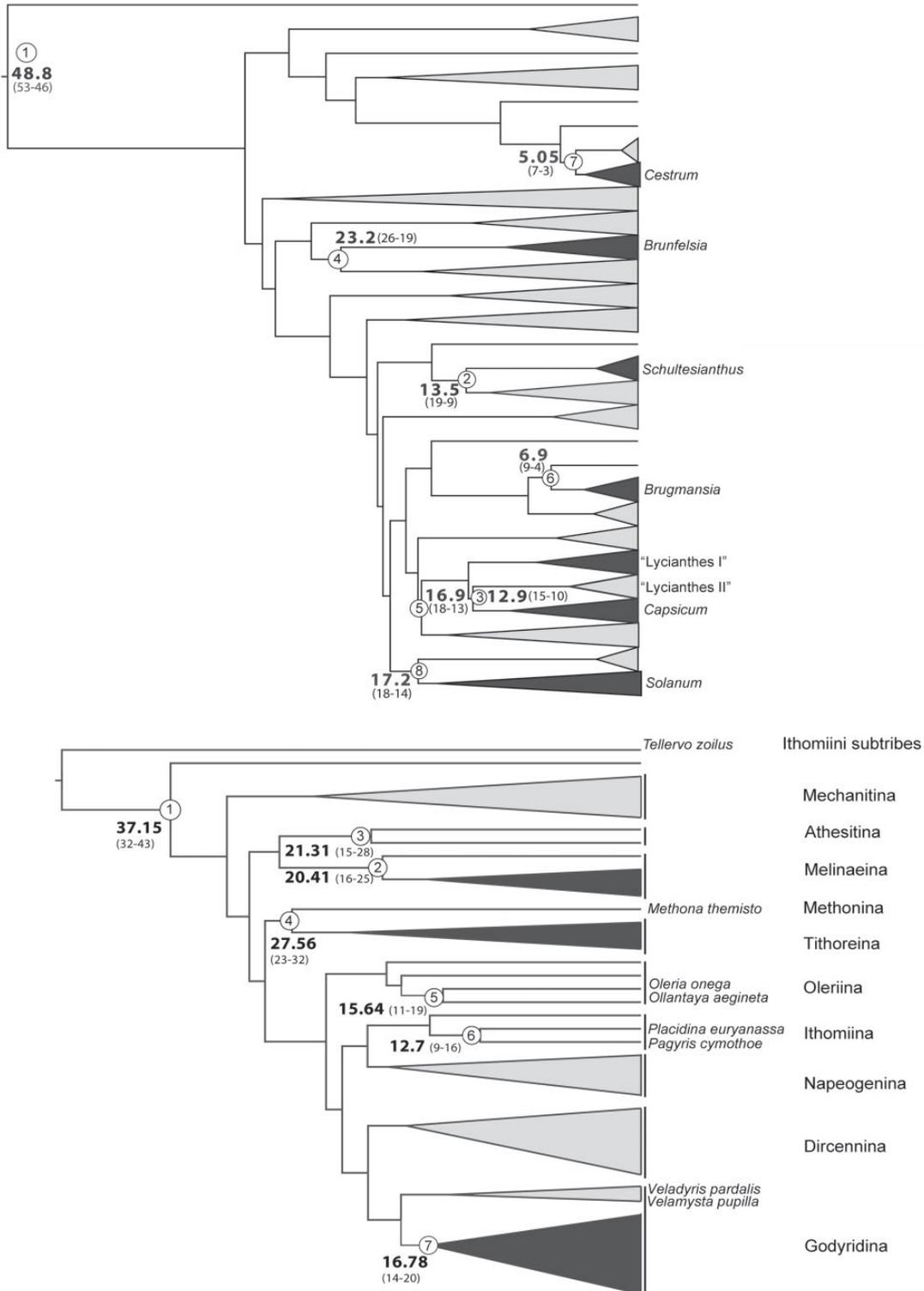
FIGURE 1.   Sources of the two sets of calibrations used in this study. Top: reduced phylogenetic tree for Solanaceae modified from Särkinen et al. (2013) featuring 8 secondary calibrations; bottom: reduced phylogenetic tree for Ithomiini featuring 7 secondary calibrations extracted from W09. In both cases, numbers in bold are mean node ages, 95% HPD intervals are shown underneath. Horizontal axes of the two trees are not proportional to clade ages.

times calculated for members of Nymphalidae extracted from W09's timetree (hereafter "ithomiine calibrations"). Regardless of which set of calibrations is used, unless stated otherwise both sets of analyses used the same

data set (hereafter "baseline data set"). This data set comprises the molecular data (COI, *wingless* and EF-1α; 3940 bp) from B14 (88 taxa; 85 Ithomiini, 1 Tellervini, 2 Danaini) with morphology excluded in

an attempt to keep our data as similar as possible to the W09 analysis (we ran a preliminary test to see if including/excluding the morphological characters had a significant effect on divergence times, which it did not: see S1 available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad.b77d0).

Sources of the Calibrations

Because inferred age estimates used as calibrations are only as valid as the original data used to obtain them (Graur and Martin 2004), and because lepidopterists have been accused before of naïvely accepting estimated ages of angiosperms (Heads 2012), we will discuss how our two calibration data sets were originally produced.

Nymphalidae.—W09 used 7 nymphalid fossils and the estimated crown ages of 6 hostplant clades distributed broadly across Nymphalidae to estimate the ages of divergence in their tree (which included 399 ingroup taxa). None of these fossils fall within Ithomiini, all range in age from 20 to 34 Ma, and each is identified as either a member of an extant genus or a close relative of an extant genus in another subfamily: 2 Libytheinae, 3 Nymphalinae, 1 Biblidinae and 1 Satyrinae. Thus the inferred ages within Ithomiini represent extrapolations from fossils of rather remotely related taxa. W09's plant calibrations provided maximum ages for 6 clades (Danainae, Biblidinae, Satyrinae, Heliconiinae + Nymphalinae and two clades within Nymphalinae), based on the ages of putative ancestral hostplant orders or families, assuming oligophagy and parsimonious ancestral hostplant reconstructions. The calibration placed closest to Ithomiini was the inferred age of Gentianales, the hypothetical ancestral hostplant order of Danainae (and the presumably plesiomorphic larval host of the ithomiine subtribe Tithoreina), at 83 Ma. Because hostplant calibrations provide only maximum ages, this is extremely conservative: the inferred ancestral hostplants of Danainae can be more precisely identified as the family Apocynaceae, which are estimated to be no older than 54 Ma (Rapini et al. 2007), substantially younger than W09's estimate for Gentianales. Therefore, this hostplant calibration was in practice uninformative.

Solanaceae.—In order to calibrate their densely sampled 1075-terminal Solanaceae tree, S13 reviewed all Solanaceae fossils found in the literature (initially 50). After excluding fossils that lacked characters to assign them reliably to particular clades within Solanaceae, 32 fossils (mostly seeds) were kept and grouped to produce two calibration points. Because more specific diagnostic features could not be observed, S13 assigned the ages of the oldest Solanaceae fossils to the stem node of the family instead of to less inclusive clades within the family and they warned that this placement might bias the analysis toward younger inferred ages. Despite this, the S13 estimates for the stem (49 Ma; HPD 46–54) and crown (30 Ma; HPD 26–34) ages of Solanaceae overlap the ranges of the stem (59 Ma; HPD 49–74) and crown (37–38 Ma; HPD 26–49) ages obtained in Bell et al.'s (2010) timetree for angiosperms.

Divergence Time Analyses using Hostplant Calibrations

Calibration details.—For these analyses we used 8 calibrations from the inferred ages of selected Solanaceae clades (Fig. 1 top) (S13). We selected every case in which complete or almost complete oligotrophy of an ithomiine clade on a clade of Solanaceae has been recorded (Willmott and Freitas 2006; K. Willmott, unpublished data), and used the stem ages of hostplant clades as calibrations (potential limitations of individual calibrations are described in the "Discussion" section) Assuming that a butterfly clade could be younger but not older than its obligate larval hostplant group, we constrained these calibrations as maximum ages and modeled them under priors that followed a normal distribution but had their upper limit truncated to middle ages (thus preventing the age of the butterfly clade from being older than the mean hostplant calibration age), and a birth–death speciation model. Because we considered the age of the most recent common ithomiine ancestor to be the most reasonable empirical optimization of the time when the butterflies began feeding on a specific hostplant, our baseline analysis (see below) placed the plant calibrations at the node of the most recent common ancestor of the butterflies feeding on that hostplant (hereafter crown age placement). The 8 hostplant calibrations and their standard deviations are shown in Table 1.

Baseline analysis and variations.—The baseline data set was run under the following combination of parameters (which accordingly we called baseline analysis): 8 calibrations from selected clades of Solanaceae (Table 1) were placed as crown ages for corresponding butterfly clades that feed on them; these priors were modeled under a normal distribution with truncated upper bounds (butterflies cannot be older than their hostplants); and using birth–death as the speciation model. However, in order to test the effects of different analytical settings and variations in the data set, we ran a number of additional controlled analyses. We compared this baseline analysis to the following variations (Fig. 2):

(1) *Including more outgroups* We compared the results from our baseline divergence times analysis to a data set that included 114 taxa, representing nearly all the genera in the subfamily Danainae (85 Ithomiini, plus 1 Tellervini and 28 Danaini from Brower et al. 2010) ("danaine data set" hereafter).

(2) *Birth-Death versus Yule as the speciation model* Leaving all the other parameters the same as in the baseline analysis, we conducted a divergence time analysis using Yule instead of birth–death as the speciation model.

TABLE 1. Ithomiine (from Wahlberg et al. 2009) and host plant-based (from Särkinen et al. 2013) calibrations used in this study

	Ithomiine calibrations	Age (SD)	Hostplant calibrations	Age (SD)
1	Ithomiini	37.15 (3.1)	Stem age of Solanaceae	48.80 (1.87)
2	Root of Melinaeina	20.41 (2.3)	Stem age of *Schultesianthus*	13.50 (2.52)
3	Root of Athesitina	21.31 (2.9)	Stem age of *Capsicum*	12.90 (1.2)
4	Root of Methonina	27.56 (2.3)	Stem age of *Brunfelsia*	23.20 (1.79)
5	Root of *Ollantaya* + *Oleria*	15.64 (2.0)	Stem age of *Lycianthes*	16.95 (1.17)
6	Root of *Placidina* + *Pagyris*	12.7 (1.6)	Stem age of *Brugmansia*	6.95 (1.54)
7	Root of Godyridina excl *Velamysta* and *Veladyris*	16.78 (1.0)	Stem age of *Cestrum*	5.05 (1.0)
8			Stem age of *Solanum*	17.21 (1.77)

Note: Each Ithomiine clade on the left feeds on the corresponding Solanaceae clade to the right (Mean values are given in millions of years, followed by standard deviations (SD) in parentheses. Numbers in the first column correspond to numbered nodes in Figure 1.

(3) *Stem versus crown placement of hostplant calibrations* As noted above, we consider crown-age placement to be the most empirically defensible assumption for hostplant calibrations, but in this analysis we explored the alternative possibility that the shift to a particular hostplant is what led to the split of the ancestor of the butterfly group in question from its sister taxon, and accordingly we ran a comparison with the calibration at the node where divergence from its sister group occurred (stem age placement).

(4) *Unconstrained normal distribution of the priors versus normal distribution with upper bounds truncated* In order to assess the effect of the upper bound truncations on inferred divergence times we ran an analysis with the priors modeled under a nontruncated normal distribution. Such an assumption allows the butterflies to be older than their hostplants, and therefore violates the premise of parsimonious coevolutionary oligophagy.

Divergence Time Analysis using Ithomiine Calibrations

Calibration details.—W09's nymphalid timetree included 41 ithomiine taxa, the inferred ages of which we employed as an alternative set of calibrations for the same baseline data set analyzed with hostplant calibrations. Following the results of empirical explorations of various dating schemes (Ho and Phillips 2009; Sauquet et al. 2012; Duchêne et al. 2014), we chose 7 age estimates corresponding to the same nodes calibrated with the Solanaceae dates (the clade containing species feeding on *Solanum* was not monophyletic in W09 so we did not obtain an eighth ithomiine calibration point for this node). In all cases, these ithomiine secondary calibrations were used as priors modeled on a normal distribution and run under a Yule speciation model following W09's parameter settings. The mean age and standard deviation of the ithomiine calibration points are shown in Table 1, and their locations within Ithomiini are indicated on the corresponding section of the W09 timetree (Fig. 1 bottom).

Analysis.—The divergence time analysis using ithomiine calibrations was conducted on a fixed topology matching the topology obtained from hostplant calibrations. We constrained the topology after noticing in preliminary analyses that the ithomiine calibrations yielded a topology slightly different from the tree obtained with hostplant calibrations Because we were mainly concerned with divergence times, we did not investigate this effect of the topology further; however, Ho and Phillips (2009) have thoroughly explored the causes of such effects.

Additional Tests

We conducted the following tests to assess the effects of including different numbers of genes and taxa, and to assess branch length variation independent of age constraints. The data and the calibrations used are described for each analysis.

Effect of more sequence data.—To ensure comparability of our baseline data set (regardless of the source of the calibrations) to W09's data set which included data from 10 gene regions, we reran the W09 data set (429 taxa) including only sequences from the same three gene regions (mtDNA COI-COII, *wingless* and Ef-1α) employed in this study. We then used W09's 11 calibrations and model parameters (uniform priors, Yule as speciation model, more details in W09) to compare age estimates from this analysis to the published results from W09.

Taxonomic sampling.—We ran an analysis with all taxa from B14 (88 taxa), and with a reduced taxon sample replicating that of W09 (44 taxa: 41 ithomiine + 3 outgroups), to assess the effect of different taxonomic sampling densities on the divergence times of selected clades of Ithomiini We used the same ithomiine calibrations, leaving the topology unconstrained.

Effect of branch lengths on divergence times.—We produced uncalibrated Maximum Likelihood (ML) trees for

the 44 taxa data set and for the 88 taxa data set using GARLI 2.0 (Zwickl 2006) to further explore the effect of branch lengths on the estimation of divergence times. We then compared the branch lengths of selected clades of these uncalibrated trees versus the divergence times estimated in our previous analysis.

Details of BEAST Analyses

We used PartitionFinder 1.1.1 (Lanfear et al. 2012) to identify the optimal partition scheme (S2 available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad.b77d0) for the data according to the Bayesian Information Criterion, which
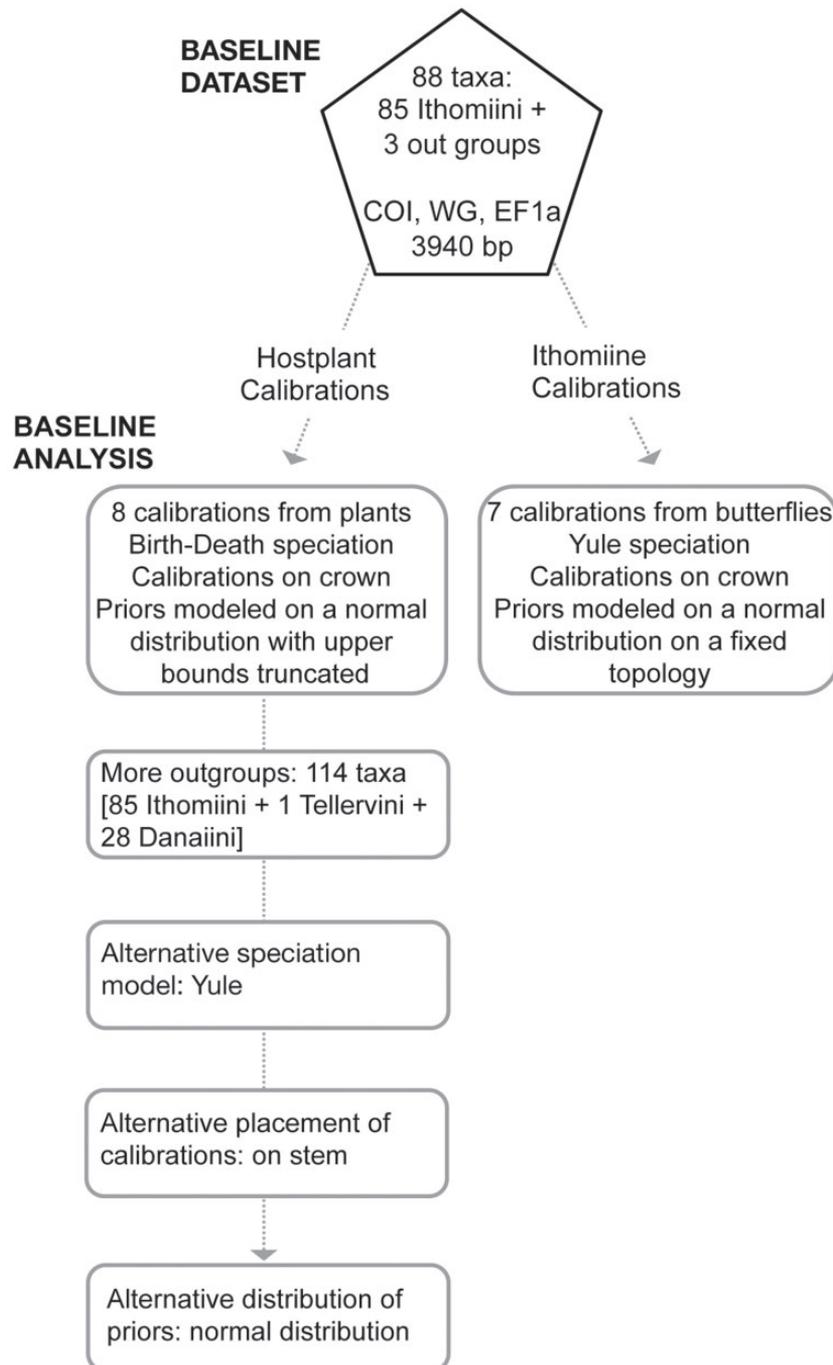
FIGURE 2. Analyses and data sets used in this study. The pentagon at the top represents our "baseline data set" this data set was used in the analyses of divergence times with hostplant and with ithomiine calibrations. Box on the left represents our "baseline analysis" the analytical variations derived from it are featured in each rectangle under it.

penalizes extra parameters the most, thus preventing overparameterization in the models. These partitions (and their corresponding optimal molecular models) were used as unlinked site models in BEAUti 2.1.3 (Bouckaert et al. 2014). In BEAST 2.1.3, a relaxed molecular clock using an uncorrelated log-normal model was applied to the baseline data set with the speciation tree prior set to the settings of each particular analytical variation. In all cases we had to conduct several Markov Chain Monte Carlo analyses that included chains of different lengths ranging from 30 million to 100 million generations (sampling parameters every 1000 steps) and the log and tree files from these runs were later combined using LogCombiner 2.1.3 (Bouckaert et al. 2014) to ensure an effective sample size (ESS) of at least 100 in all the parameters. Tracer 1.6 (Rambaut et al. 2014) was used to examine the ESS of the different parameters and to define the "burn in". TreeAnnotator 2.1.3 (Bouckaert et al. 2014) was used to generate a maximum clade credibility topology (MCCT) of all the sampled trees, with node heights representing mean heights. Finally, FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/, accessed December 2014) was used to visualize and edit the topologies.

Comparisons among Alternative Data sets and Analytical Assumptions

Because our sampling of Ithomiini is more comprehensive than W09's, when comparing our results to theirs we focused on the mean crown ages of major clades in Ithomiini (subtribes). We compare our age estimates after calibrating with the hostplant and with ithomiine ages to the original W09 age estimates within sections 1 and 2 of the "Results," respectively, and to each other in section 3. We quantified the absolute differences in divergence times (in myr) and assessed whether the 95% credibility intervals of age estimates obtained using alternative calibrations overlapped or not.

RESULTS

Calibrating with Hostplant Divergence Times

Results of baseline analysis.—The mean crown age of Ithomiini was estimated to be 31 myr (95% highest posterior density (HPD) 23–39) (Table 2). The youngest subtribe is Godyridina with a mean crown age of 7.7 myr (HPD 6-10) and the oldest subtribe is Mechanitina, with a mean crown age estimated at 13.1 myr (HPD 11–15). According to our results, Methonina is at least 4 but not older than 21 myr old (upper limit of the 95% HPD from our oldest estimate). The significantly older estimate for Methonina from W09 must have been affected by the inclusion of only one species, making the estimate *ipso facto* a stem age. In all cases, the mean crown age of the subtribes were younger than estimates obtained by W09 (on average 11 myr, or 50%, younger).

Figure 3 shows the maximum credibility tree obtained from our baseline analysis, the placement of the calibrations and the mean crown ages of ithomiine subtribes. Means and HPD intervals for every node of this tree are shown in S3 (available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad.b77d0).

Results of analytical variations.—The results of these variations and how they compare to the results from the baseline analysis are illustrated in Figure 4. Using the danaine data set (i.e., when more outgroups were included), the mean crown age of the 10 subtribes was estimated to be 5 myr older than in the baseline analysis. The same applies for the inferred age of Ithomiini itself, which was estimated to be 12 myr older than in the baseline analysis. Using Yule instead of birth–death as the speciation model produced similar results: on average, the subtribes were 4 myr older and the mean crown age of Ithomiini was 14 myr older than in the baseline analysis. In contrast, placing the calibrations on the stem instead of crown nodes resulted in a younger mean age for Ithomiini (12.7 myr younger), and younger

TABLE 2. Mean crown ages (in millions of years, with 95% HPD intervals) of major Ithomiine clades obtained in our baseline analysis (with hostplant calibrations) and in our divergence time analysis using Ithomiine calibrations

Node	Baseline analysis	Min	Max	Ithomiine calibrations	Min	Max	W09	Min	Max	HPD overlapping
Ithomiini*	31.0	23	39	34.0	31	38	37.2	32	43	Yes
Melinaeina*	12.5	10	14	23.9	21	27	20.4	16	25	No
Athesitina*	11.5	9	13	22.5	18	27	21.3	15	28	No
Methonina*	10.6	7	13	17.2	13	21	27.6	23	32	Yes
Tithoreina	11.1	8	14	24.7	20	29	23.0	18	28	No
Mechanitina	13.1	11	15	26.2	22	30	25.9	22	30	No
Oleriina*	11.8	10	14	20.9	18	24	20.1	16	24	No
Ithomiina*	9.2	7	11	18.2	16	21	16.7	13	20	No
Napeogenina	10.3	8	12	20.8	18	23	20.0	17	23	No
Dircennina	9.4	8	12	18.9	16	21	18.5	16	22	No
Godyridina*	7.7	6	10	19.0	17	21	19.0	16	22	No

Note: The original estimates obtained by Wahlberg et al. (2009) are shown for comparison ("W09"). Whether or not HPD intervals overlap between these analyses is shown by the column to the right. Asterisks denote clades that contain a calibration.
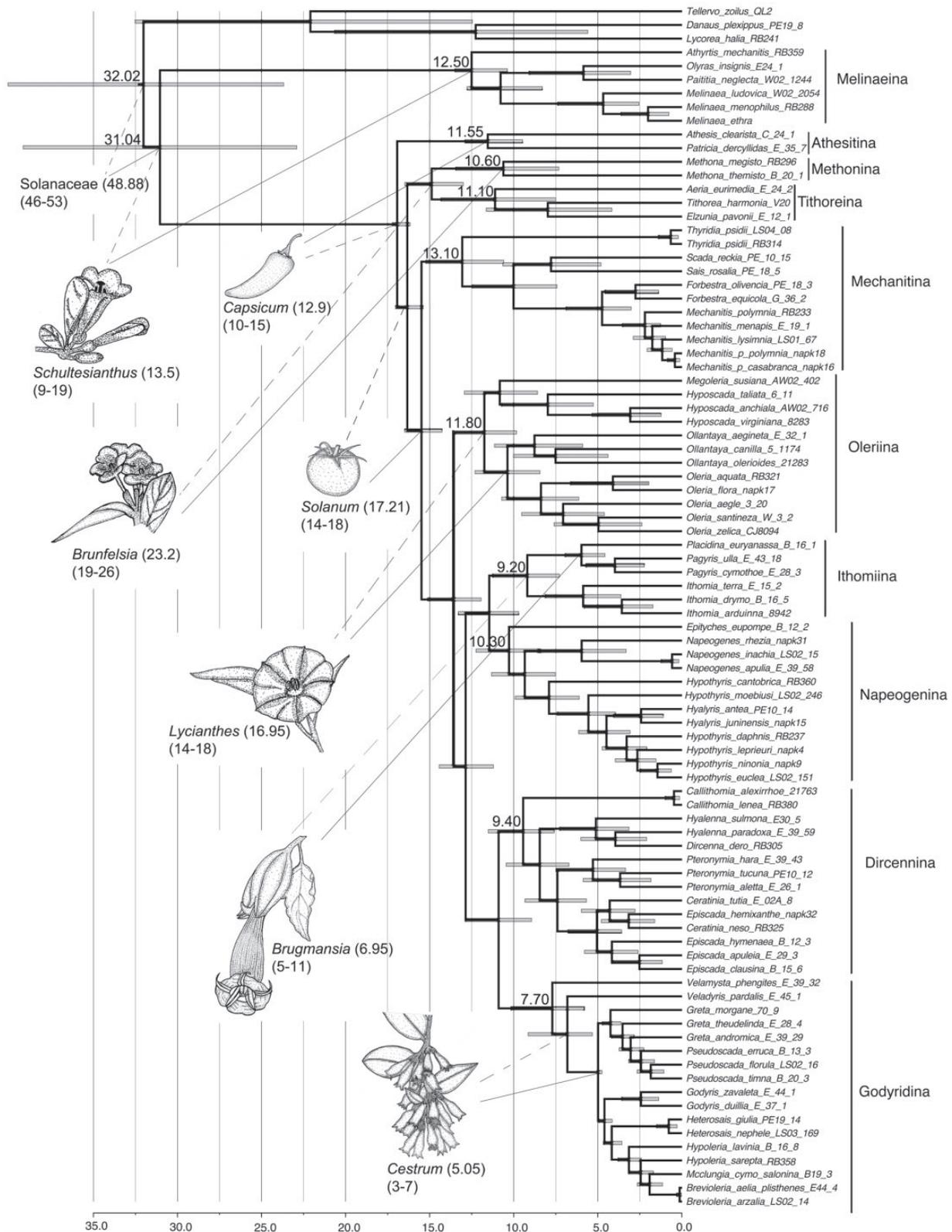
FIGURE 3.   BEAST maximum credibility tree with divergence times for Ithomiini estimated from 8 calibrations extracted from Särkinen et al. (2013) based on the crown ages of selected Solanaceae genera (named hostplant calibrations in text). Mean crown ages for each subtribe are shown with bars representing the 95% HPD; the plant taxon chosen for calibration and its alternative placement either at the crown (solid line) or stem (dotted line) of the dated group are shown.
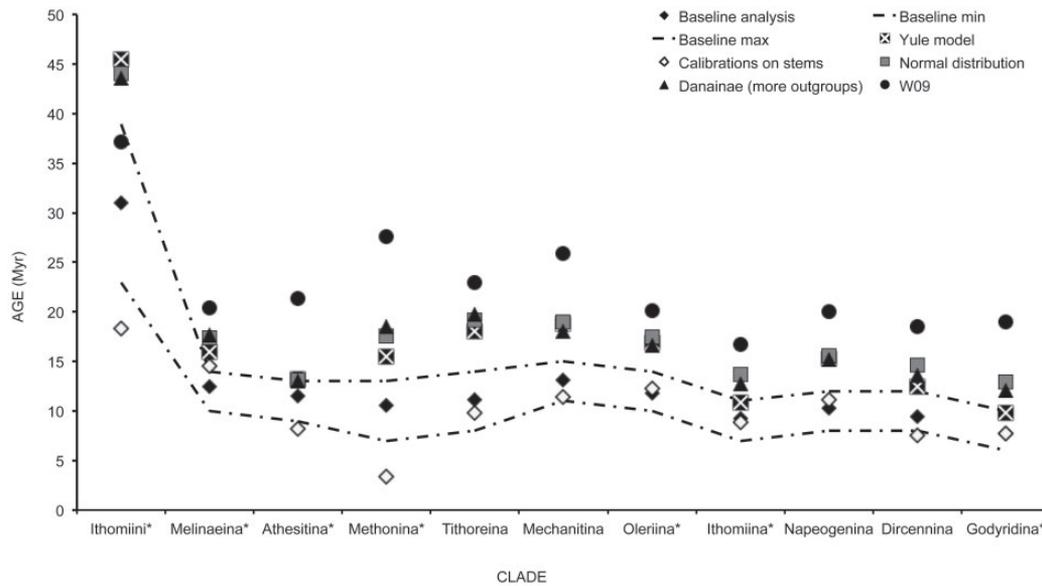
FIGURE 4. Effect of different analytical settings on mean divergence times of Ithomiini and subtribes inferred using hostplant calibrations. The dashed line marks the min and max values of the 95% HPD for the baseline analysis. For comparison purposes, mean age estimates obtained by Wahlberg et al. (2009) are featured in solid black circles.

mean crown ages for 6 of the 10 subtribes than in the baseline analysis. These 6 subtribes were on average 2.6 myr younger. When the priors were modeled under a normal distribution, the ages of all the subtribes were older (on average 5 myr older) than in the baseline analysis, as well as the mean crown age of Ithomiini (13 myr older). In most cases, the 95% HPDs of these variations overlapped with the 95% HPD of the baseline analysis, except for the ages of Ithomiini and the subtribe Methonina.

Calibrating with W09's Ithomiine Divergence Times

Calibrating the same topology obtained in the results above using 7 ithomiine calibrations estimated the crown age of Ithomiini to be 34 myr (HPD 30.5–38) (Table 2) (Fig. 5). The ages of the 10 ithomiine subtribes ranged from 26 to 17 myr (mean = 21 myr). These estimates are on average 2 myr different from comparable clades estimated by W09, and the maximum difference was 10 myr (the mean crown age of Methonina). We provide means and HPD intervals for every node on this tree in S4 (available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad.b77d0).

Age Estimates using Ithomiine Versus Hostplant Calibrations

Age estimates of the major clades inside Ithomiini using secondary calibrations from ithomiines were on average twice as old as estimates using hostplant calibrations (Table 2). The 95% credibility intervals for ages of the major clades overlapped between the analyses in only two instances (the crown age of Ithomiini and the age of the subtribe Methonina).

Results from Additional Tests

Effect of more sequence data.—The differences in the mean divergence times between the W09 original dating analysis with 10 genes and our reanalysis of their data set using only the 3 genes used by B14 are mostly very small (<1 myr; S5 available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad.b77d0). Some subtribes in our reanalysis are slightly younger than in W09, others are slightly older. Only Melinaeina and Athesitina were estimated to be some 5 myr older by the 3 genes alone than by the full W09 data set. These results are in accord with previous findings (Rannala and Yang 2007; Duchêne et al. 2014) suggesting that sequence length does not affect molecular-clock estimates very much and we therefore do not discuss this variable further. A table comparing the divergence times obtained in all the above analyses is provided as S6 (available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad.b77d0).

Taxonomic sampling.—Four of the 10 subtribes were older in our taxonomically more complete data set (B14) (on average 3 myr) than in our analysis with a reduced sample (and therefore also older than W09 estimates) (Fig. 5). Overall, reducing the data set to replicate W09's taxonomic sampling produced estimates equal to (estimated crown age for Ithomiini was 37.16 Ma (95% HPD 33–41 Ma) the same age obtained by W09 (37.15 Ma)) or very close to W09's results.

Effect of branch lengths on divergence times.—To eliminate the possibility that the difference in the divergence times reported above between the W09 data set with 44 taxa and B14 with 88 occurs simply because
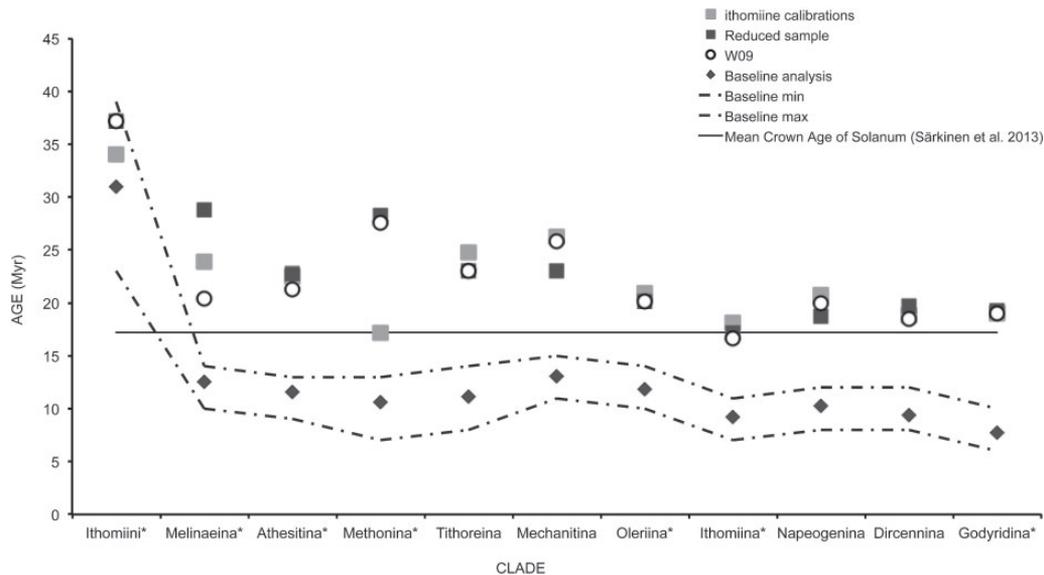
FIGURE 5.   Effect of different analytical settings on mean divergence times of Ithomiini and subtribes inferred using ithomiine calibrations. Results of the baseline analysis (diamonds, and min and max 95% HPD) and Wahlberg et al. (2009) original estimates (black circles) are shown for comparison. The mean crown age of *Solanum* obtained by Särkinen et al. (2013) is featured as a solid black line (see text).

the additional taxa from B14 happen to have longer branches, we compared branch lengths of terminals from the B14 and W09 taxon samples (uncalibrated topologies for each data set can be found as S7 and S8 available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad.b77d0). A twotailed t-test ($t = 1.23$, $df = 63$, $P = 0.22$) failed to find significant differences between the mean branch lengths of the W09 sample and the mean branch length of the added taxa, suggesting that the effect on the age estimates is due to the number of taxa, rather than to consistent differences in branch length among the samples. S8 (available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad.b77d0) shows the branch lengths (added branch paths from root to tips) of ithomiine subtribes plotted against estimated divergence times for the same clades.

DISCUSSION

Ithomiine Versus Hostplant Calibrations and Coevolutionary Interactions

The close association between butterflies and their angiosperm hostplants has been documented many times (Brues 1920; Brower and Brower 1964; Ehrlich and Raven 1965; Futuyma and Agrawal 2009), and the effect of the interaction on their reciprocal diversification has been implicated (Thompson 1994; Wahlberg et al. 2013). Though coevolution does not always entail cospeciation (e.g. Percy et al. 2004), parsimony suggests that the age of an oligotrophic herbivore clade should not be older than the age of its hostplant clade (Janz and Nylin 1998); this has been the rationale for using crown ages of plants as maximum calibration points for butterfly clades (as in W09 and other butterfly studies).

Brown (1987) hypothesized that Ithomiini must have colonized Solanaceae well after the generic diversification of the plant family in the New World, which implies that Ithomiini reached their current diversity through ecological speciation facilitated by the availability of novel niches present within *Solanum* and other Solanaceae lineages (Willmott and Freitas 2006). Implicit in this hypothesis is the assumption that Solanaceae are older than Ithomiini. However, based on ithomiine calibrations, estimates for both the stem (38.12 myr, HPD 33–44) and crown ages (34.01 myr, HPD 30–38) of Ithomiini are considerably older than the S13 estimate of Solanaceae's crown age (30.5 myr, HPD 26–34), whereas our plant-based calibrations put the crown age of Ithomiini around 31 myr (HPD 23–39). Furthermore, in almost every case (with the exception of Methonina), ithomiine calibrations produced age estimates for ithomiine subtribes that are older than the apparent ages of their hostplant clades (Table 2 and Figs. 1, 2 and 5).

If the ithomiine-calibrated ages are correct, the inferred age of *Solanum* by S13 represents another temporal conundrum. Within the species-rich subtribes Mechanitina, Oleriina, Ithomiina, Napeogenina, Dircennina and Godyridina, the use of a hostplant other than *Solanum* is rare, making *Solanum* the parsimonious ancestral hostplant of these clades (Willmott and Freitas 2006), and feeding on anything else the likely result of a host shift. However, based on ithomiine calibrations (Fig. 5), the age of the most recent common ancestor (MRCA) of the clade containing these 6 subtribes (~30 myr; the equivalent clade in W09 is dated ~24.8 myr) is almost double that of the putative age of *Solanum* (crown age ~17 myr). If this age of *Solanum* is accurate, then these results would completely falsify the principle of parsimony on which the use of hostplant
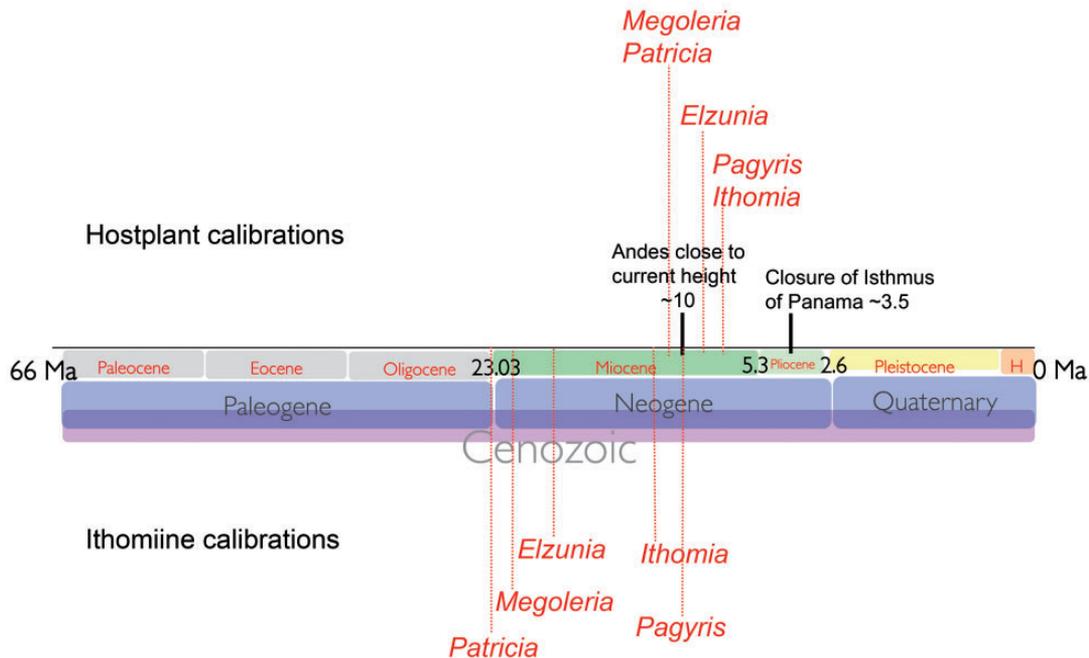
FIGURE 6. Comparison of mean crown age estimates for montane genera in Ithomiini according to hostplant (top) and ithomiine (bottom) calibrations.

ages to calibrate herbivore clades is based; namely, if the *Solanum*-feeding Ithomiini are really 30 myr old (as suggested by ithomiine calibrations), then at least 20 ithomiine lineages that were extant at that time must have colonized their hostplants independently, instead of the single colonization implied if the clade evolved after the appearance of *Solanum*. Of course we could explain this pattern by independent colonizations; however once we admit this, the rationale for using the crown ages of hostplants as maximum age calibrations for the butterflies is vitiated.

When independent calibrations provide congruent estimates of the age of a taxon, confidence that the hypothesis is valid grows. On the other hand, when there is significant disagreement among alternatives, at least one must be wrong, leading to reconsideration of the methods and assumptions underlying the calibration process. The results using hostplant calibrations fit much better with the herbivore-hostplant coevolutionary narrative, but this is a circular argument, because the timing of butterfly divergences is dependent on the ages of the hostplant clades under that calibration scheme. Is there additional, independent evidence that might help us select among these discordant dating scenarios?

Ithomiine Versus Hostplant Calibrations: Concordance with Biogeography?

Fortunately, in the case of Ithomiini, there are scenarios of diversification in relation not only to larval hostplants, but also to the timing of the geological events leading to formation of the Andes.

The tropical Andes began to rise initially in the south, with high elevations being attained progressively later to the north. Although the Andes likely exceeded 1000 m in elevation in Bolivia and southern Peru by 20 Ma, similar elevations were achieved in the Ecuadorian and Colombian Andes only in the last 10 myr or so (Gregory-Wodzicki 2000). Ithomiine calibrations imply that several entirely montane genera in Ithomiini diverged from their sister taxa prior to these orogenic events (Fig. 6). These clades include *Patricia* (23 Ma), *Elzunia* (18.7 Ma), *Pagyris* (13.3 Ma), *Megoleria* (20 Ma), *Ollantaya* (17 Ma), and *Veladyris* + *Velamysta* (20 Ma). The dates obtained using ithomiine calibrations thus either diminish the role that increasing mountain elevation played in the origin of current montane ithomiine diversity (Chazot et al. 2014), or suggest an origin for the montane ithomiine clades in the southern Andes, with subsequent northward expansion and diversification. Differences in timing of Andean uplift may explain in part why the older Andes, south of the equator, have such a diverse ithomiine fauna in comparison with the younger northern Andes, as also suggested for other butterfly taxa (Casner and Pyrcz 2010; Pyrcz et al. 2013).

In contrast, hostplant calibrations place the diversification of all montane ithomiine genera much more recently (Fig. 6), cutting both W09's ages and our ithomiine-calibrated ages by half or more, and temporally fitting better the hypothesis of the uplift of the northern Andes as an agent of diversification. Indeed, Andean uplift has been argued as an important event affecting diversification in a variety of butterfly groups, including *Hypanartia* (Willmott et al. 2001), ithomiine genera (Whinnett et al. 2005; Jiggins et al.

2006), *Heliconius* (Brower 1996), and Phyciodina (Wahlberg and Freitas 2007). Elias et al. (2009) dismissed the role of the Andes in the initial diversification of *Ithomia* and *Napeogenes* (both ithomiine genera) based on W09 dates because all sister taxa occurring on opposite sides of the Andes had diverged before 3.9 Ma, but younger ages would imply that Andean orogeny actually was involved in the radiation of these two genera, as well as in the rapid diversification of members of the species-rich and predominantly high elevation *Oleria makrena* species group (de Silva et al. 2010).

Other Studies Where W09's Estimates are Used as Secondary Calibrations

As mentioned in the introduction, W09's dates have been used as secondary calibrations in multiple studies, some of which have expressed difficulty in interpreting the outcomes of their dating analyses. For example, Mullen et al. (2011) found it hard to explain the colonization of South America by the lowland *Adelpha* clade prior to the formation of the Isthmus of Panama, given a ~11 myr divergence time estimate and a ~3.5 myr estimate for the rise of the isthmus (but see Montes et al. 2012 and Coates and Stallard 2013).

Beyond the evolutionary implications for diversification of ithomiines and their hostplants and temporal congruence with biogeographical phenomena, we also observed that the age estimates obtained using ithomiine calibrations were generally older than W09's original estimates (Table 2, columns 2 and 3), although most of the sequence data are the same between the two studies. A major difference between the studies is their taxonomic representation: W09 sampled 41 ithomiine exemplars compared with our 85. By reducing B14 to replicate W09's taxonomic sampling, we obtained age estimates closer to theirs (black squares vs. black circles in Fig. 5). This suggests that as the number of taxa sampled increases, age estimates increase as well. Other empirical studies have found similar effects (Linder et al. 2005; Schulte 2013). Operating on the commonsense premise that more data are better, one might interpret this result to suggest that if taxon sampling is incomplete, BEAST may underestimate crown group ages. The reason for this is false inference of synapomorphy between nonsister lineages that is correctly identified as homoplasy when additional taxa are included (Bromham et al. 2002). False synapomorphies would make taxa appear more similar, and, assuming a clock, more recently diverged than they actually are. Following this line of reasoning and because B14 was also based on exemplars, we might expect that future studies with more comprehensive taxonomic representation could shift these dates further back in time.

If this is a general phenomenon, we expect that a study with more comprehensive taxonomic sampling using secondary calibrations extracted from a higher-level phylogeny, will tend to yield estimates older than dates for the same nodes in the original study.

Because divergence times are inferences regarding actual historical events, some consistency between independent (or semi-independent) estimates is expected, even allowing for error. On the other hand, our exploratory analyses suggest that increasing the number of calibrations reduces discrepancies between estimates. We noticed that even with a larger taxon sample by increasing the number of calibrations from 3 to 7, all other things being equal, the new estimates were closer to the estimates made by W09 (results not shown).

Whether W09's dates consistently overestimate divergence times across Nymphalidae can only be determined with additional analyses based on independent calibration points. The age of Nymphalidae is still far from certain, due to the poor fossil record of butterflies. The ~90 myr inferred age of Nymphalidae from W09 leaves a considerable (56 myr) gap before the first appearance of a nymphalid in the fossil record (*Libytheana vagabunda* and its sister species *L. florissanti* at 34 myr)—nearly the first two thirds of the hypothetical age of the group—with no fossil evidence at all. Furthermore, Wahlberg et al. (2013) showed that by removing a secondary 90 myr calibration (from W09) from their analysis of higher-level lepidopteran divergences, the estimated age of Nymphalidae shifts to a mean of 60 myr.

Most other butterfly timetree analyses to date (see citations above) have used W09 age estimates as secondary calibrations, because independent tests of the divergence ages for other groups of nymphalids are contingent on the availability of alternative evidence to provide new calibrations, as we have done here. However, since 2009, botanical studies have provided additional, in some cases younger, crown age estimates for the plant groups used by W09 as calibrations (S10 available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad.b77d0). Given how sensitive dating analyses seem to be to the calibration of nodes, it is difficult to predict how a revised tree for Nymphalidae using these alternative dates would look. Judging from our results and given that W09's dates has been used as a baseline for many other studies, a reassessment of W09's original age estimates would be interesting and desirable.

Conclusions and Perspective

We have shown that the timing of ithomiine diversification calibrated with ages of Solanaceae clades differs substantially from previous estimates based on an alternative set of secondary calibrations from nymphalid butterflies. If we accept the ages of hostplants as maximum ages of the butterfly clades that feed on them, then prior estimates of butterfly clade ages based on fossils and more inclusive hostplant clades appear to be too old. Alternatively, if W09's dates are correct, historical factors traditionally considered important in promoting the diversification of ithomiines, such as

hostplant shifts and the uplift of the Andes, appear irrelevant because both are temporally inconsistent with the ages of the butterfly clades. On the other hand, the younger ages for ithomiines estimated here using hostplant calibrations are more consistent with ideas that orogenic events and hostplant shifts during adaptive radiations have been important in ithomiine diversification. Of course, these conclusions and, more generally, the results of our analyses, are contingent upon the accuracy of Solanaceae age estimates, which are also subject to error (e.g. Wilf and Escapa 2014).

It has been known for some time (Sanderson 1990; Hugall and Lee 2007) that branch length estimates depend upon taxon sampling. The so-called "node density effect" (Bromham et al. 2002) results from detection of homoplasy mistakenly inferred to be homology by the addition of taxa that break up long branches. This phenomenon has been discussed at length in the context of relative estimates of branch length in different parts of trees with sparser or denser taxon sampling (e.g., Venditti et al. 2006, 2008; Hugall and Lee 2007; Venditti and Pagel 2008). However, it seems to have been overlooked in the molecular clock literature, in which absolute branch length is used as an indicator of time since divergence.

Our results show that branch lengths and corresponding age estimates over the entire tree vary with the completeness of taxon sampling when calibrations and other model parameters are held constant: the addition of new paths to the tree changes the inferred lengths (and corresponding ages) of preexisting paths (Fig. S8 available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad. b77d0). These observations raise important questions about relaxed clock models. Do current models adequately account for unobserved character change on unsampled branches? Are node ages inferred in such studies logically valid as secondary calibrations for more densely sampled data sets when those age estimates would have been different if the taxa in the denser sample had been included in the original analysis?

The initial motivation of our study was not to provide yet another exploration of the effects of priors on age estimates in dating studies. Instead we were motivated by the evolutionary paradox that previous age estimates for Ithomiini were older than the inferred ages of their hostplants. Our study employed two separate sets of secondary calibrations to infer ages for clades of ithomiine butterflies. In general, because there is no way to know whether parameters have been adequately specified to match the evolutionary vagaries of the data, the only available test of a model-based clock's success is congruence among independent estimates. The inferred ages we obtained from alternative sets of calibrations are strongly incongruent, so at least one of the estimates is wrong. This finding led us to the more general observation that although an individual analysis may propose relatively modest credibility intervals around the estimated age of a given clade, when viewed as a set of equally plausible alternative

hypotheses, the ranges of credibility of the ages across analyses are enormous (Table 2). For example, credible ages for Ithomiini range from 43 to 23 Ma, and for Mechanitina from 30 to 11 Ma. In addition, the credibility intervals of alternative estimates are in many instances nonoverlapping (Table 2).

Despite the conflicting scenarios implied by our results, we remain optimistic regarding the future of dating studies. As divergence times become available for different hostplant groups we will be able to test alternative hypotheses, as we have done in this study. Indeed, although we feel it would be premature to discard the estimates for Ithomiini produced by W09, we have now two alternative timing hypotheses that can be tested both by compatibility with past knowledge and ability to accommodate future discoveries (e.g., fossils). The challenge of choosing among hypotheses like these should generate inspiring discussions about how can we test a given dating hypothesis. Offering explicit predictions of the kinds of data that could be used to test the results of dating analyses seems to us key for these studies to remain a hypothesis-driven endeavor.

SUPPLEMENTARY MATERIAL

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.b77d0.

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