# Revisiting the Insect Mitochondrial Molecular Clock: The Mid-Aegean Trench Calibration

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## Abstract

Phylogenetic trees in insects are frequently dated by applying a "standard" mitochondrial DNA (mtDNA) clock estimated at 2.3% $My^{-1}$, but despite its wide use reliable calibration points have been lacking. Here, we used a well-established biogeographic barrier, the mid-Aegean trench separating the western and eastern Aegean archipelago, to estimate substitution rates in tenebrionid beetles. Cytochrome oxidase I (*cox1*) for six codistributed genera across 28 islands (444 individuals) on both sides of the mid-Aegean trench revealed 60 independently coalescing entities delimited with a mixed Yule-coalescent model. One representative per entity was used for phylogenetic analysis of mitochondrial (*cox1*, 16S rRNA) and nuclear (Mp20, 28S rRNA) genes. Six nodes marked geographically congruent east–west splits whose separation was largely contemporaneous and likely to reflect the formation of the mid-Aegean trench at 9–12 Mya. Based on these "known" dates, a divergence rate of 3.54% $My^{-1}$ for the *cox1* gene (2.69% when combined with the 16S rRNA gene) was obtained under the preferred partitioning scheme and substitution model selected using Bayes factors. An extensive survey suggests that discrepancies in mtDNA substitution rates in the entomological literature can be attributed to the use of different substitution models, the use of different mitochondrial gene regions, mixing of intraspecific with interspecific data, and not accounting for variance in coalescent times or postseparation gene flow. Different treatments of these factors in the literature confound estimates of mtDNA substitution rates in opposing directions and obscure lineage-specific differences in rates when comparing data from various sources.

**Key words:** molecular clock calibration, Bayesian relaxed clock, insects, mitochondrial DNA rates, Tenebrionidae, mid-Aegean trench.

## Introduction

Evolutionary scenarios frequently rely on estimates of node ages in time-calibrated phylogenetic trees. Where fossils are unavailable, as is the case for many groups of insects, a standardized rate of molecular change may be applied to obtain such estimates. For insect mitochondrial DNA (mtDNA), the most widely quoted rate of molecular evolution is Brower's (1994) calibration based on a set of seven studies that provided age estimates of lineage splits ranging from 300 to 3,250,000 years ago. Regression on uncorrected pairwise distances against inferred number of years since lineage divergence revealed a surprisingly strong linear relationship with $y = 2.34 \times 10^{-6}x$ and $r^2 = 0.996$, for a substitution rate of 0.0115 per site per My equal to 2.3% divergence. Although Brower's (1994) rate appears to be satisfactory to estimate ages in many groups, its uniformity in the seven original studies is surprising given the mixed use of protein-coding and ribosomal markers, whose rates are expected to differ greatly. In addition, mutation rates in recently diverged haplotypes at or near the population level have now been found to be much higher than phylogenetic rates (Ho et al. 2005, 2007), possibly due to the delayed effect of purifying selection. This "time dependency" of the clock may affect a period of up to ~1 Ma, well within the time window from which most of Brower's (1994) calibration points were drawn.

Subsequent estimates of insect mtDNA substitution rates based on biogeographic vicariance, island ages, fossils, or other independent evidence (supplementary table S1, Supplementary Material online), reported both decreased (Sperling et al. 1997; Pruser and Mossakowski 1998; Andersen et al. 2000) or elevated (Fleischer et al. 1998; Luchetti et al. 2005; Shapiro et al. 2006) rates, which is generally attributed to lineage-specific or gene-specific effects (e.g., a lower rate of 1.5% divergence in cytochrome oxidase I [*cox1*]; Farrell 2001). However, the effects of the methodology used for rate or age estimation have not been fully appreciated. Recently developed "relaxed-clock" methods, which allow substitution rates to vary among branches either in an autocorrelated or uncorrelated manner (Sanderson 2002, 2003; Thorne and Kishino 2002; Yang 2004; Drummond et al. 2006), hold great promise for a more accurate calibration of the mtDNA clock and its rate variation among insect lineages. Yet, accurate estimations of substitution rates require careful choice of the model of sequence evolution used to correct for multiple hits (Yang 1996; Arbogast et al. 2002), an issue that has received little attention in the entomological literature for its effect on

clock estimates. Moreover, the preponderance of biogeographic and paleoecological data used to calibrate the clock requires well documented geological or paleoclimatic evidence and a demonstration that the assumed barriers constitute a true obstacle to dispersal for the focal group. Finally, several factors may confound the correlation between geological time and lineage divergence, such as ancestral polymorphism (Edwards and Beerli 2000) or postseparation gene flow that delay lineage sorting along an assumed biogeographic boundary (Carstens and Knowles 2007).

Improved estimates of the mtDNA clock in insects therefore require further study of systems that are less affected by frequently encountered sources of error. The mid-Aegean trench in the eastern Mediterranean is highly suited for this kind of analysis. This geological formation originated in the Upper Miocene (12–9 Mya) and led to the initial split of the united landmass of Agäis (Creutzburg 1963; Dermitzakis and Papanikolaou 1981), separating the western and eastern Aegean archipelago (fig. 1). These areas have remained subdivided by the surrounding ocean ever since, except for the Messinian desiccation of the Mediterranean basin from 5.96 to 5.33 Mya (Krijgsman et al. 1999) when a land bridge may have existed via a deep rift valley.

The Aegean islands harbor species-rich assemblages of darkling beetles (Coleoptera: Tenebrionidae) highly suited for biogeographic clock calibrations. Many species are flightless with limited dispersal capabilities, in particular, the "geophilic" lineages associated with ecologically stable soil types. Genetic variation in a few of these species has already been shown to be highly structured geographically and is deeply subdivided along the mid-Aegean trench (Papadopoulou, Anastasiou, et al. 2009). Codistributed lineages of tenebrionids therefore can be used to calibrate rates simultaneously in multiple taxon pairs that were subjected to a single geological event, thus reducing stochastic effects of lineage sorting and variation in substitution rates (Edwards and Beerli 2000; Hickerson et al. 2003). A comprehensive survey of genetic variation of six flightless, geophilic tenebrionid genera with wide distribution throughout the Aegean established several temporally congruent lineage splits that were attributable to the formation of the mid-Aegean trench. This provides an independent calibration of the mtDNA clock in a major lineage of insects and permits an assessment of the sensitivity of inferred substitution rates to model selection, alternative partitioning schemes, the use of relaxed versus strict clock methods, and gene-specific differences in rates between protein-coding and rRNA genes. Comparisons with literature data show that discrepancies in rates found by different studies can be attributed largely to methodological issues, although substitutions rates in the Aegean tenebrionids may still be higher than most existing estimates for insects.

## Material and Methods

### Taxon Sampling and DNA Sequencing

A total of 444 specimens from six flightless, geophilic tenebrionid genera were sampled from 11 to 25 islands each, and 51–122 individuals were sequenced per genus (table 1, supplementary table S2, Supplementary Material online). Total genomic DNA was extracted from thorax or leg tissue using the Promega 96-well plate kit. An 826–829 bp fragment of the 3′ end of the *cox1* gene was amplified using primers C1-J-2183 (Jerry) and TL2-N-3014 (Pat) (Simon et al. 1994) or JerryTen and PatTen (Papadopoulou, Anastasiou, et al. 2009). A 513–524 bp fragment of the single-copy nuclear muscular protein 20 (Mp20) locus, including 469 bp of coding region and one intron, was amplified using the primer pair Mp205′ and Mp203′ (Pons et al. 2004) for Pimeliinae or Mp20Trib5′ and Mp20Trib3′ for Tenebrioninae (Papadopoulou, Anastasiou, et al. 2009). A 433–437 bp portion of the mitochondrial 16S rRNA gene (*rrnL*) was amplified using LR-N-13398 (16Sar) (Simon et al. 1994) and LR-J-12961 (16Sb2) (Cognato and Vogler 2001), and a 646–655 bp fragment of the nuclear 28S rRNA gene was amplified using 28SFF and 28SDD (Monaghan et al. 2007). Amplification products were purified using Millipore Multiscreen 96-well plates (Millipore, Billerica, MA) and sequenced in both directions using the BigDye technology and an ABI PRISM 3700 DNA Analyzer (Applied Biosystems). Sequence chromatograms were assembled and edited using the Sequencher 4.6 software (Gene Codes Corporation, Ann Harbor, MI). *Cox1* and Mp20 sequences for the genera *Dailognatha*, *Eutagenia*, and *Zophosis* are from Papadopoulou, Anastasiou, et al. (2009). New sequences have been submitted to the EMBL Nucleotide Sequence Database (supplementary tables S2–S3, Supplementary Material online).

### Species Delimitation, Alignment, and Phylogenetic Analysis

To minimize the effect of increased mutation rates at the intraspecific level (Ho et al. 2005, 2007), sequence variation was divided into within- and between-species groups using the generalized mixed Yule-coalescent (GMYC) model (Pons et al. 2006; Fontaneto et al. 2007). This procedure identifies a threshold value for the shift in branching rate from coalescent lineage branching to interspecific diversification on an ultrametric tree and delimits "independently evolving" mtDNA clusters. The analysis was carried out using the R package SPLITS (SPecies LImits by Threshold Statistics) available at http://r-forge.r-project.org/projects/splits/ with the "single-threshold" option. A clock-constrained tree required for the analysis was built separately for the full *cox1* data sets of each genus, after removal of identical haplotypes. Tree searches were performed with Bayesian analysis in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), applying separate models for two partitions (1st and 2nd codon positions together vs. 3rd codon position) as selected by Akaike information criterion (AIC) in MrModeltest 2.2 (Nylander 2004), with two parallel runs of 5 million generations each and using one cold and two incrementally heated Markov chains ($\lambda = 0.1$) and sampling every 1,000 steps. Trace plots were visually inspected, and convergence diagnostics (standard deviation [SD] of split frequencies, effective sample size), as implemented in

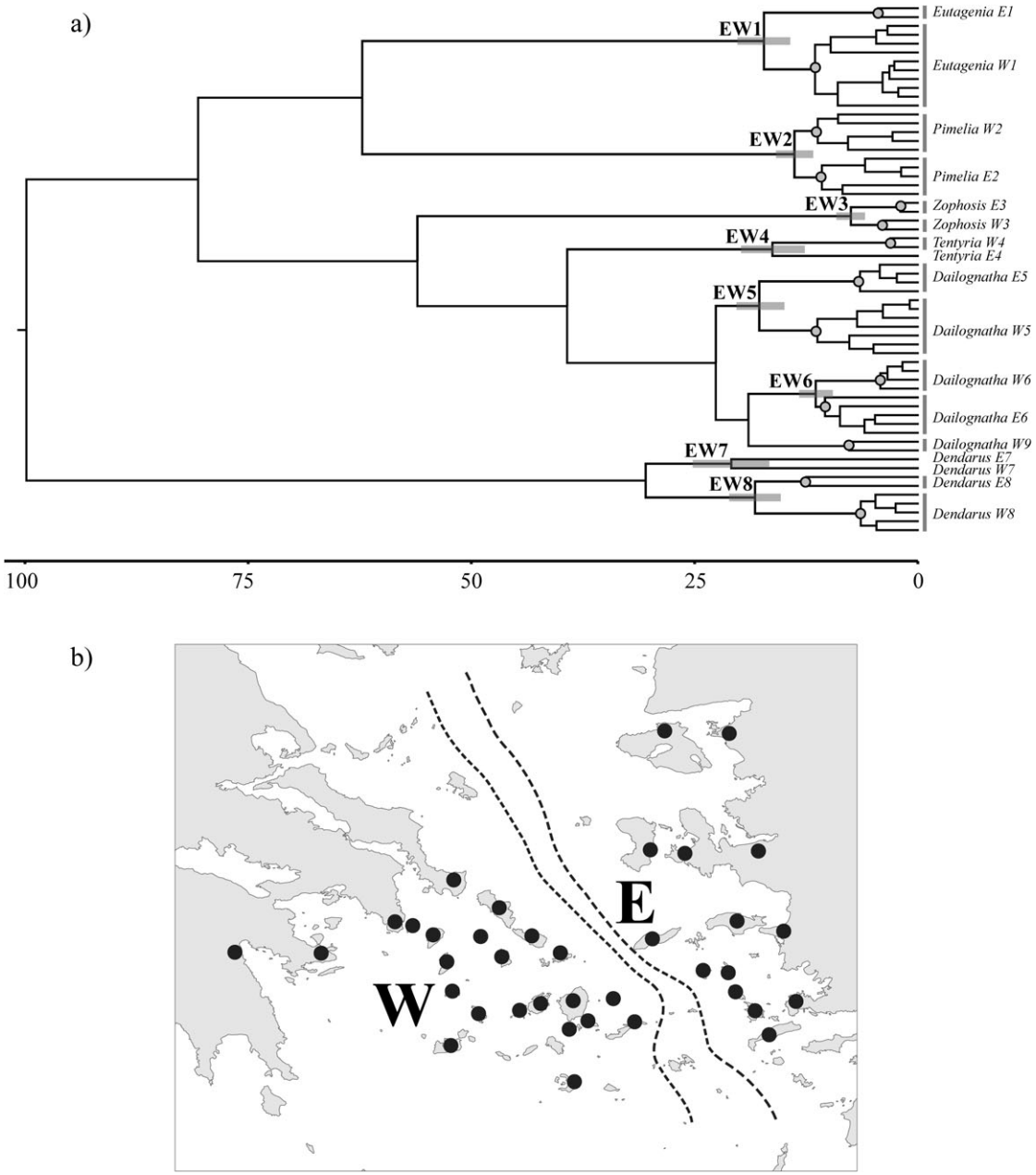


FIG. 1. (*a*) Relatively dated four-gene tree (combined data set of *cox1*, *rrnL*, 28S, and Mp20) of six tenebrionid genera generated using an uncorrelated lognormal clock in BEAST. Eight east–west nodes (EW1–EW8) are compared for temporal congruence (using a relative scale 0–100). Node heights correspond to mean values across 9,000 post burn-in trees, whereas gray bars indicate ± 1 SD for the eight focal nodes (see table 2 for values). (*b*) Map of the central Aegean region showing the sampled islands and mainland regions on the east and on the west of the mid-Aegean trench, dashed lines represent the presumed position of the trench.

MrBayes and Tracer 1.4.1 (Drummond and Rambaut 2007), were checked to ensure that the Markov chain had reached stationarity. After discarding the first 2.5 million generations as burn-in, trees were summarized using an "all-compatible" consensus. Each consensus tree was converted to ultrametric using penalized likelihood as implemented in r8s 1.7 (Sanderson 2003) with the optimal smoothing parameter selected by cross-validation of values between 0.01 and 1,000 following the procedure described in the r8s manual.

For the phylogenetic analysis at the species level, a single exemplar representing each GMYC group in the *cox1* analysis was selected for sequencing of three additional markers given above. A range of different alignment strategies was assessed for the length-variable *rrnL*, 28S, and the intron region of Mp20. We compared five gap penalty combinations (gap opening penalty: 5-6.66-10-15-20 vs. gap extension penalty: 6.66) in ClustalW (Thompson et al. 1994) and three different iterative refinement methods (E-INS-i, G-INS-i, and L-INS-i) in MAFFT 6.240 (Katoh et al. 2005; Katoh and Toh 2008). Each resulting alignment was assessed for congruence with the unambiguously aligned protein-coding regions of *cox1* and Mp20 using the

**Table 1.** Sampled Taxa and Results of the GMYC Model for Species Delimitation.

| Taxon | Tribe | sp[a] | isl[b] | Sequences[c] | Entities[d] | Clusters[e] | $logL_{Null}$[f] | $logL_{GMYC}$[g] | T (E-W)[h] |
|---|---|---|---|---|---|---|---|---|---|
| *Dailognatha*[i] | Tentyriini | 2 | 25 | 122 | 23 (21–32) | 18 (16–20) | 160.9728 | 174.6146*** | 22 (9–13) |
| *Dendarus* | Dendarini | 7 | 12 | 51 | 10 (8–18) | 9 (7–13) | 5.6441 | 11.1804* | 9 (3–6) |
| *Eutagenia*[i] | Stenosini | 1 | 18 | 63 | 12 (11–17) | 10 (8–12) | 28.4019 | 34.4749** | 12 (2–10) |
| *Pimelia* | Pimellini | 1 | 11 | 56 | 12 (9–13) | 11 (9–12) | 13.1996 | 18.2333* | 10 (5–5) |
| *Tentyria* | Tentyriini | 1 | 18 | 59 | 2 (2–5) | 2 (2–4) | 8.4808 | 18.3449*** | 3 (1–2) |
| *Zophosis*[i] | Zophosini | 1 | 25 | 93 | 8 (3–16) | 5 (3–11) | 102.1494 | 114.2980*** | 4 (2–2) |

[a] Number of morphologically described species.
[b] Number of islands or mainland regions sampled per taxon.
[c] Number of *cox1* sequences used to apply the GMYC model.
[d] Total number of independent entities identified by the GMYC model including singletons (range of entities within 2 logL of the model).
[e] Number of entities with more than one individual.
[f] The likelihood of the null model.
[g] The likelihood of the GMYC model, likelihood ratio test ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.
[h] Number of terminals per genus in the six-genera phylogenetic tree sampled from the east or the west of the mid-Aegean trench.
[i] Genera that were analyzed in Papadopoulou, Anastasiou, et al. (2009).

Incongruence Length Difference (ILD) test (Farris et al. 1994). Tree lengths were calculated using parsimony searches in PAUP* 4.0b10 (Swofford 2002) with 1,000 random addition sequence replicates and gaps treated as "5th state." We separately selected the alignment strategy for each locus that gave the lowest score for the ratio ILD/length of the combined-analysis tree (Wheeler and Hayashi 1998) with respect to the protein-coding regions and then tested the overall congruence of the resulting data set (partitioned as *cox1*—*rrnL*—28S—Mp20 exon—Mp20 intron) using the partition homogeneity test in PAUP* with 100 replicates.

Parsimony analysis of the combined data set was performed in PAUP* with 1,000 random addition sequence replicates and gaps treated either as 5th state or as "missing data," and nonparametric bootstrap was conducted with 1,000 pseudoreplicates. We used PhyML 3.0 (Guindon and Gascuel 2003) to perform unpartitioned maximum likelihood (ML) analysis under a general time reversible (GTR)+Γ+I substitution model and calculate bootstrap support values with 100 replicates. We also carried out partitioned ML searches with RAxML 7.0.4 (Stamatakis 2006) under seven different partitioning schemes (supplementary table S4, Supplementary Material online). The GTRMIX model was employed, so the initial tree searches were conducted with the GTRCAT approximation but the final tree topology was evaluated under a separate GTR+Γ+I model for each partition. In total, 1,000 bootstrap replicates were performed for each partitioning scheme using the rapid RAxML bootstrapping algorithm (Stamatakis et al. 2008).

We tested the null hypothesis that all pairs of east/west clades are reciprocally monophyletic and sister to each other, comparing parsimony and RAxML searches under that topological constraint with those of unconstrained searches and assessing the significance of the observed differences with the Shimodaira–Hasegawa test (Shimodaira and Hasegawa 1999) as implemented in PAUP*. We applied a single GTR+Γ+I model (as PAUP* does not permit applying a separate substitution model to each partition) with parameters estimated by PhyML, and the significance of the test was evaluated using RELL sampling with 10,000 replicates.

## Relative Node Ages and Clock Calibrations

Node ages and substitution rates were estimated using an uncorrelated lognormal relaxed clock in BEAST 1.4.8 (Drummond et al. 2006; Drummond and Rambaut 2007). In all analyses, the among-genera relationships and the monophyly of the eastern and western clades were constrained according to the results of the topology tests conducted on the four-gene data set. This was considered necessary because otherwise, if a small proportion of the sampled trees from the posterior distribution did not include all eastern and western clades as reciprocally monophyletic, the 9–12 Mya calibration would have been partially assigned to incorrect nodes. Relative node ages were estimated by fixing the root node to an arbitrary value (normal prior distribution with a mean of 100 and SD of 1). Two independent runs of 50 million generations (sampling every 5,000th generation) were performed for each analysis, using a Yule tree prior and the default options for all other prior and operator settings. The convergence and mixing of each Markov chain Monte Carlo chain was assessed by inspection of the trace plots and the effective sample sizes using Tracer 1.4.1 (Drummond and Rambaut 2007). Samples from both independent runs were then pooled after removing a 10% burn-in using LogCombiner 1.4.8. The means and standard errors (SEs) of the node heights were summarized using Tracer, and SDs were calculated by multiplying the SEs by the square root of the effective sample size in each case.

Estimates of substitution rates were performed under an uncorrelated lognormal relaxed clock in BEAST as described before, but instead of fixing the root node, a normal prior distribution was applied on the ages of the selected calibration points, with a mean of 10.5 My and SD = 1.5 (0.05 quantile: 8.033, 0.95 quantile: 12.97), to reflect the geological age of the Aegean trench at 9–12 Mya. We estimated rates for the combined *cox1* and *rrnL* data set under an Hasegawa-Kishino-Yano (HKY) model, a GTR model, a GTR+Γ+I, and four partitioning schemes (P1: *cox1* vs. *rrnL*, P2: 3rd codons vs. all other sites, P3: 1st and 2nd positions vs. 3rd positions vs. *rrnL*, P4: 1st vs. 2nd vs. 3rd vs. *rrnL*). We applied a separate substitution

model to each partition as selected by the AIC implemented in MrModeltest 2.2 (Nylander 2004) (supplementary table S5, Supplementary Material online). Moreover, we used the same priors on the node ages and models selected by the AIC to estimate rates for each of the five gene regions separately (*cox1*, *rrnL*, Mp20 exon, Mp20 intron, 28S), with *cox1* treated either as a single partition or divided by codon position.

A strict clock was applied for comparison using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) under a uniform prior on branch lengths. All searches were conducted with 5 million generations and two parallel runs using one cold and two incrementally heated Markov chains ($\lambda = 0.1$) and sampling every 1,000 steps, and the first 2.5 million generations were discarded as burn-in. Mean node heights and 95% higher posterior limits across all post burn-in trees were calculated using TreeAnnotator 1.4.8, and these numbers were converted to % divergences $My^{-1}$ assuming that the most recent node corresponds to separation at a minimum of 9 Mya and the oldest node at a maximum of 12 Mya.

We used Bayes factors to assess the ML of different models and partitioning schemes. The harmonic mean of the sampled likelihoods was estimated either using the "sump" command in MrBayes or, in the case of the BEAST searches, by Tracer with 1,000 bootstrap replicates. For the interpretation of the Bayes factors, we followed the widely used cutoff values proposed by Kass and Raftery (1995), when comparing partition schemes that required equal numbers of free parameters. When partition schemes differed in total number of free parameters, we calculated the ratio ln(Bayes Factor)/$\Delta$p ($\Delta$p = difference in number of total free parameters between alternative partition schemes) and used the recommendations of Pagel and Meade (2004), as applied by Miller et al. (2009), which suggest at least a 10 lnL increase in the harmonic mean per additional free parameter before accepting a more complex model.

MEGA4 (Tamura et al. 2007) was used to calculate average pairwise uncorrected and Kimura 2-parameter distances between each east/west pair, and distances were converted to maximum and minimum divergences per My, corresponding to 9 and 12 Mya, respectively.

## Results

### Phylogenetic Analysis of *cox1* and mtDNA Cluster Delimitation

The *cox1* Bayesian trees of *Dendarus*, *Pimelia*, and *Tentyria* (supplementary figs. S1–S3, Supplementary Material online) and *Dailognatha*, *Eutagenia*, and *Zophosis* (Papadopoulou, Anastasiou, et al. 2009) revealed strong phylogenetic clustering and geographical structure. The GMYC model had a significantly better fit to the data than the null model of uniform coalescent branching for all lineages ($P < 0.05$) and identified between 2 (*Tentyria*) and 23 (*Dailognatha*) GMYC entities (table 1 and supplementary figs. S1–S3, Supplementary Material online; Papadopoulou, Anastasiou, et al. 2009). The number of GMYC groups greatly exceeded that of Linnaean names, as most of these highly subdivided lineages are currently described as a single species (table 1). Each of the GMYC clusters was geographically restricted to a single island or a group of adjacent islands either on the eastern or western side of the mid-Aegean trench. A notable exception was the eastern GMYC cluster in the genus *Tentyria* found on the volcanic island of Santorini located to the west of the mid-Aegean trench (supplementary fig. S3, Supplementary Material online). This may be attributed to a recent recolonization event, after the last catastrophic eruption of the volcano 3,500 years ago, a pattern that has been suggested for many other taxa (Thornton 2007). In total, 67 entities (55 clusters and 12 singletons) were recognized at the point of the highest likelihood of the GMYC model. One individual per cluster and selected singletons were chosen for sequencing of the *rrnL*, Mp20, and 28S markers. Confidence intervals for the number of clusters were calculated (table 1), but we selected exemplars to match the ML solution, except in the case of *Tentyria*, where we used the suboptimal solution of three GMYC clusters (A1, A2, and B in supplementary fig. S3, Supplementary Material online). After failure to sequence two of the chosen individuals, the final four-gene data set comprised 60 terminals, including 22 eastern and 38 western lineages (fig. 1 and supplementary table S3, Supplementary Material online).

### Alignment and Phylogenetic Analysis

Alignment parameters for each length-variable gene region were selected to reduce incongruence with the protein-coding regions (supplementary table S6, Supplementary Material online). The resulting concatenated matrix was highly congruent among the five partitions ($P = 0.98$; partition homogeneity test). Parsimony and ML analyses supported the monophyly of each genus, whereas the topology (*Dendarus*, ((*Dailognatha*, *Tentyria*), *Zophosis*), (*Eutagenia*, *Pimelia*)) was favored by all searches except for parsimony when gaps were treated as 5th state (supplementary table S4, Supplementary Material online). Within each genus, most of the eastern (E) and western (W) lineages were reciprocally monophyletic, with generally high bootstrap support (fig. 1; EW1–8), except for clades E6 and W6 (*Dailognatha*) that were unresolved with respect to each other (supplementary table S7, Supplementary Material online). Tree scores from searches constrained for monophyly of all eight east/west pairs and their reciprocal monophyly were very similar to those from unconstrained analyses (supplementary table S8, Supplementary Material online), and when scores were slightly lower these differences were not significant in the Shimodaira–Hasegawa test under parsimony (all $P >> 0.05$) nor ML ($P > 0.05$; supplementary table S9, Supplementary Material online). These results provide justification to conduct mtDNA clock calibration on trees constrained for the monophyly of these nodes.

### Testing for Contemporaneous Divergence

The eight east/west pairs were tested for temporal congruence using a relative dating approach (Loader et al. 2007), by comparing their relative ages under a relaxed-clock model based on all four genes or only on the mtDNA data

**Table 2.** Relative Age Estimates (mean age ± 1 SD) for Eight East–West Nodes, When Fixing the Root Node to 100 Under an Uncorrelated Lognormal Relaxed Clock in BEAST, using Either the Four-Gene Data set or Only the mtDNA Data set (combined *cox1* + *rrnL*).

| | Four genes | mtDNA |
|---|---|---|
| **EW1**[a] | **14.48–20.21** | **21.24–28.61** |
| **EW2** | **12.01–15.88** | **17.26–22.49** |
| **EW3** | **6.08–9.16** | **8.89–12.94** |
| **EW4** | **12.92–19.87** | **18.05–26.27** |
| **EW5** | **15.15–20.59** | **22.85–30.27** |
| **EW6** | **9.83–13.29** | **14.27–18.94** |
| **EW7** | **16.89–25.22** | **23.2–32.73** |
| **EW8** | **15.54–21.16** | **21.1–28.02** |

[a] Bold letters indicate the six nodes with largely overlapping ranges that were selected as calibration points.

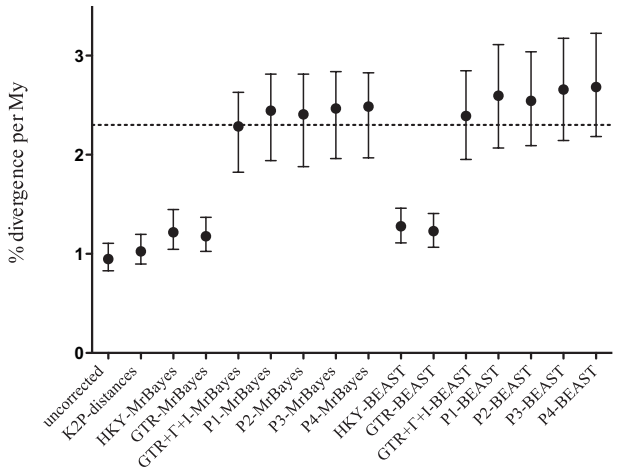


**Fig. 2.** Estimated rates of divergence for the combined *cox1* + *rrnL* data set based on six calibration points corresponding to the age of mid-Aegean trench (9–12 Mya) and using either pairwise distances (uncorrected or Kimura 2-parameter), a strict clock in MrBayes or a relaxed lognormal uncorrelated clock in BEAST under different nucleotide substitution models (HKY, GTR, GTR + Γ + I, and four partitioning schemes P1–P4 as described in the text). Error bars correspond to the 95% highest posterior density limits (BEAST and MrBayes analyses) or to the range of values calculated across 6 calibration points (*P* distances). The dashed line indicates the standard 2.3% per My rate (Brower 1994).

set. Mean values of node ages were within ± 1 SD of each other for six east/west pairs (table 2), supporting a hypothesis of contemporaneous divergence. The two remaining east/west pairs were either marginally (*Dailognatha*, EW6) or significantly (*Zophosis*, EW3) more recent (table 2). In light of the geological history of the region, the six contemporaneous nodes were attributed to the formation of the mid-Aegean trench at 9–12 Mya, whereas the EW3 node was assumed to reflect the younger east/west subdivision after the end of the Messinian desiccation at 5.96 Mya. The EW6 split may also be associated to the initial formation of the mid-Aegean trench but affected by postseparation dispersal (e.g., an eastern sample was found on Amorgos island immediately to the west of the trench), and the E6/W6 separation was poorly supported in the phylogenetic analysis. Therefore, only the six temporally congruent east/west nodes were employed as calibration points based on the age of the mid-Aegean trench.

## Calibration of Substitution Rates Based on the Mid-Aegean Trench Geological Age

Substitution rates for the combined *cox1* + *rrnL* data set on trees calibrated at 9–12 Mya for the six nodes revealed great differences depending on the substitution model used for correction. The estimated divergences per My ranged from 1% when using uncorrected distances and approximately 1.2% when using a HKY or a GTR model without accounting for rate heterogeneity among sites. When applying a GTR+Γ+I model, this divergence estimate increased to 2.23% (strict clock in MrBayes) or 2.39% (relaxed clock in BEAST) without partitioning and to 2.69% when using the P3 or P4 partitioning scheme in BEAST (fig. 2). The differences in estimated rates among partitioning schemes were consistent between MrBayes and BEAST analyses (fig. 2) but were in all cases higher by 0.1–0.2% with the latter. Bayes factor comparisons favored the P3 partitioning scheme in both MrBayes and BEAST analyses when applying the ln(Bayes Factor)/Δp ≥ 10 criterion or the P4 partitioning scheme applying the Kass and Raftery (1995) criterion of 2lnBF ≥ 10 (table 3), in either case supporting a rate of 2.7% (relaxed clock; BEAST) or 2.5% (strict clock; MrBayes). Applying a strict clock in BEAST under the preferred P3 partitioning scheme gave an estimated rate of 2.6% and Bayes factors comparisons between the strict and the uncorrelated lognormal relaxed clock favored the relaxed clock (lnBF = 18.32). Our estimates for *cox1* on its own were higher than the average of the two mitochondrial genes (3.36% unpartitioned or 3.54% for the preferred partitioning scheme), whereas they were lower for *rrnL* (1.06%). For the nuclear genes, we estimated mean divergence rates of 3.68% $My^{-1}$ for the intron region of Mp20, 0.66% $My^{-1}$ for the exon, and 0.12% $My^{-1}$ for 28S (table 4).

The use of an uncorrelated relaxed-clock approach, as implemented in BEAST, also permits comparisons of the clocklikeness in different gene regions (Drummond et al. 2006) and measurement of the degree of rate autocorrelation among lineages (Ho 2009). Our results indicate that *cox1* evolves in a more clock-like manner than *rrnL* (lower ucld.mean and coefficient of variation; table 3), which is in agreement with findings of other studies (Gaunt and Miles 2002). Both mitochondrial regions do not deviate greatly from the strict clock, whereas all nuclear regions (including the presumably neutrally evolving intron) showed much greater variation in rates among branches, as the SD of branch rates and the coefficient of variation are both greater than 1 (table 4). In terms of autocorrelation of rates between neighboring branches, we only found a significantly positive covariance in the case of the Mp20 exon (table 4).

## Applying the "Standard" Insect Mitochondrial Clock

When the mean of the branch rates on the combined *cox1* and *rrnL* data was fixed at 0.0115, that is, 2.3%

**Table 3.** Bayes Factor Comparisons for Selection of Substitution Model and Partitioning Scheme. a) MrBayes Analyses Under a Strict Clock b) BEAST Analyses Under an Uncorrelated Lognormal Relaxed Clock.

| | HME[a] | HKY | GTR | GTR+Γ+I | P1 | P2 | P3 | P4 |
|---|---|---|---|---|---|---|---|---|
| **a) MrBayes** | | | | | | | | |
| HKY (4)[b] | −18486.14 | — | 68.31[c] | 504.49 | 198.40 | 217.10 | 138.83 | 104.09 |
| GTR (8) | −18212.91 | 273.23[d] | — | 1376.85 | 238.43 | 262.88 | 150.58 | 108.30 |
| GTR+Γ+I (10) | −15459.21 | 3026.93 | 2753.70 | — | 31.44 | 60.34 | 39.11 | 29.01 |
| P1(21) | −15113.36 | 3372.78 | 3099.55 | 345.85 | — | n/a | 46.77 | 27.74 |
| P2(21) | −14795.46 | 3690.68 | 3417.45 | 663.75 | 317.90 | — | 17.87 | 12.61 |
| P3(32) | −14598.88 | 3887.26 | 3614.03 | 860.33 | 514.48 | 196.58 | — | 6.81 |
| P4(42) | −14530.75 | 3955.39 | 3682.16 | 928.46 | 582.61 | 264.71 | 68.13 | — |
| **b) BEAST** | | | | | | | | |
| HKY (4)[b] | −18356.50 | — | 66.49[c] | 499.19 | 192.18 | 210.09 | 134.67 | 101.25 |
| GTR (8) | −18090.54 | 265.97[d] | — | 1364.60 | 230.85 | 254.28 | 146.04 | 105.34 |
| GTR+Γ+I (10) | −15361.34 | 2995.16 | 2729.19 | — | 24.72 | 52.40 | 35.26 | 26.64 |
| P1(21) | −15089.46 | 3267.05 | 3001.08 | 271.89 | — | n/a | 45.80 | 27.65 |
| P2(21) | −14784.94 | 3571.56 | 3305.59 | 576.40 | 304.51 | — | 18.12 | 13.15 |
| P3(32) | −14585.67 | 3770.84 | 3504.87 | 775.68 | 503.79 | 199.28 | — | 7.68 |
| P4(42) | −14508.90 | 3847.61 | 3581.64 | 852.45 | 580.56 | 276.05 | 76.77 | — |

Note.—n/a, not applicable.

[a] The harmonic mean of sampled likelihoods as estimated by MrBayes or Tracer.

[b] Numbers in brackets: total number of free parameters required for each model or partitioning scheme.

[c] Above the diagonal: ln(Bayes Factor)/Δp (where Δp: difference in total number of free parameters between two models).

[d] Below the diagonal: ln(Bayes Factor).

divergence $My^{-1}$ (Brower 1994), analyses under five partitioning schemes and using a uncorrelated lognormal relaxed clock in BEAST, resulted in estimated mean ages ± 1 SD for the six contemporaneous east/west nodes that were compatible with the age of the mid-Aegean trench (table 5). The node EW3 (genus *Zophosis*) was estimated to be much more recent (4.78–4.91 Mya), an age compatible with post-Messinian divergence, whereas EW6 ranged between 7.26 and 7.65 Mya. When the 2.3% standard clock was applied to the individual mitochondrial genes, ages of the six contemporaneous east/west nodes were much higher for *cox1* (12.3–17.5 Mya) and much lower for *rrnL* (3–4.9 Mya) (table 5). The effect of data partitioning was investigated, both for the combined *cox1* and *rrnL* data set (P0–P4) and *cox1* on its own. Comparisons of the resulting ages suggested that a greater number of partitions resulted in slightly higher estimates (table 5). For example, compared with the unpartitioned data the P4 partitioning caused an increase in estimated node ages of 0.15–1.7 My (i.e., an increase by 2.8–14.4%), with the lowest percentage corresponding to the most recent node (EW3) and the highest percentage to the oldest node (EW7).

## Comparing Existing Calibrations from the Literature

An extensive literature search starting from publications citing Brower (1994) found 30 other studies (supplementary table S1, Supplementary Material online) that estimated substitution rates for insect mtDNA based on biogeographic, paleoclimatic, fossil, or other independent evidence. These data were compiled for comparisons, separately for studies that did not account for rate heterogeneity among sites (uncorrected distances or using simple substitution models) and those that used models

**Table 4.** Estimated Rates Per Gene Region Based on Six East–West Calibration Points and Using a Lognormal Uncorrelated Relaxed Clock in BEAST, Mean Values ± 1SD.

| | Mean Rate[a] | ucld.mean[b] | ucld.stdev[c] | Coefficient Variance[d] | Covariance[e] |
|---|---|---|---|---|---|
| *cox1*[f] | 0.0168 ± 0.0018 | 0.0169 ± 0.0019 | 0.2571 ± 0.0674 | 0.2609 ± 0.0702 | −0.0120 ± 0.0903 |
| *cox1* (2)[g] | 0.0177 ± 0.0019 | 0.0178 ± 0.0019 | 0.2973 ± 0.0644 | 0.3031 ± 0.068 | −0.0134 ± 0.0898 |
| 16S | 0.0054 ± 0.0009 | 0.0049 ± 0.0008 | 0.5106 ± 0.1238 | 0.5418 ± 0.1448 | 0.0001 ± 0.0895 |
| mtDNA[f] | 0.0120 ± 0.0012 | 0.0119 ± 0.0011 | 0.1863 ± 0.0619 | 0.1878 ± 0.0632 | −0.0176 ± 0.0914 |
| mtDNA (P3)[g] | 0.0133 ± 0.0013 | 0.0131 ± 0.0013 | 0.2602 ± 0.0554 | 0.264 ± 0.0578 | −0.0141 ± 0.0913 |
| Mp20 intron | 0.0184 ± 0.0152 | 0.0496 ± 0.2628 | 2.5717 ± 0.5399 | 4.4511 ± 1.1499 | 0.0781 ± 0.1136 |
| Mp20 exon | 0.0033 ± 0.0006 | 0.0024 ± 0.0008 | 1.6353 ± 0.2375 | 2.3847 ± 0.5021 | 0.2118 ± 0.1454 |
| 28S | 0.0006 ± 0.0003 | 0.8273 ± 6.2150 | 3.1057 ± 1.0567 | 5.4504 ± 1.8708 | 0.0140 ± 0.0538 |
| nDNA | 0.0017 ± 0.0003 | 0.0012 ± 0.0003 | 1.4434 ± 0.2044 | 2.0133 ± 0.4148 | 0.2058 ± 0.1387 |

[a] Number of substitutions per site divided by tree length.

[b] Mean of branch rates.

[c] The SD of the branch rates.

[d] Coefficient of variation.

[e] Covariance between parent and child branch rates.

[f] Unpartitioned.

[g] Preferred partitioning scheme.

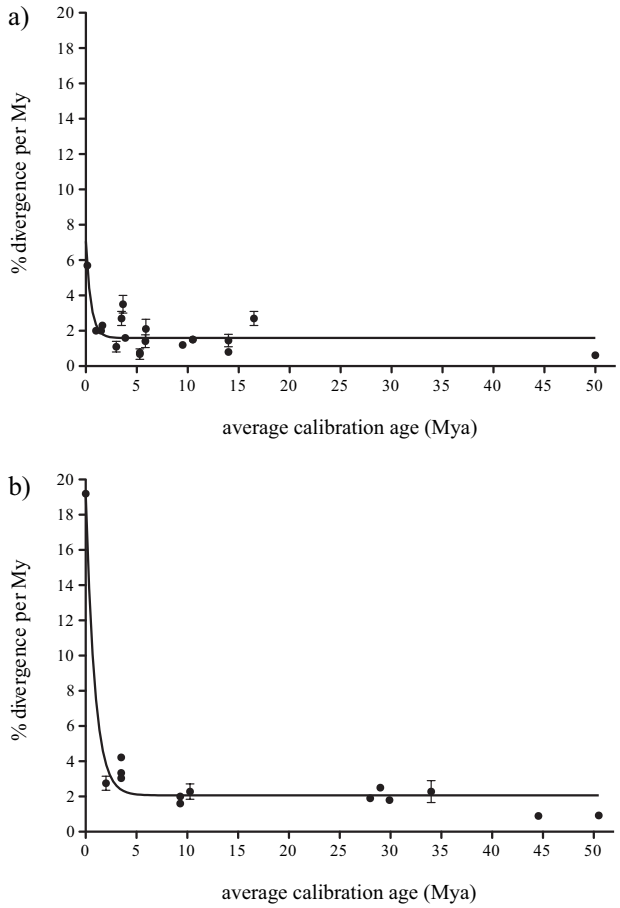


**FIG. 3.** Rates of mtDNA divergence estimated for different insect groups by 31 other studies (supplementary table S1, Supplementary Material online) plotted against the average age of the calibration points used for the estimation. Estimated rates are based either on protein-coding genes or a combination of protein-coding and rDNA or tRNA gene regions. An exponential curve with three parameters was fitted under the least-squares criterion following Ho et al. (2005). (*a*) Studies that did not account for rate heterogeneity among sites; Rate($t$) = $5.408e^{-1.794t} + 1.5231$, $R^2 = 0.60$, (*b*) Studies that accounted for rate heterogeneity among sites using a gamma distribution; Rate($t$) = $17.256e^{-1.157t} + 2.0968$, $R^2 = 0.97$.

incorporating gamma-distributed rate variation. The estimated rates obtained in these studies were plotted against the average calibration age (fig. 3), and an exponential curve with three parameters was fitted under the least-squares criterion (Rate($t$) = $\mu e^{-\lambda t} + k$). Following Ho et al. (2005), the constant $k$ represents the "long-term" substitution rate, which was $k = 1.52$ (% divergence per My) for uncorrected distances and $k = 2.10$ for the studies that had accounted for among-site rate variation. The latter showed a better fit to the exponential curve ($R^2 = 0.97$, fig. 3*a*) than the uncorrected rates ($R^2 = 0.60$, fig. 3*b*). In both cases, linear regression would result in a much poorer fit ($R^2 = 0.14$ for fig. 3*a*, $R^2 = 0.19$ for fig. 3*b*).

## Discussion

### The Importance of Model Selection

Accurate estimation of substitution rates and divergence times relies on the model of sequence evolution used to correct for multiple hits (Yang 1996; Arbogast et al. 2002). However, Brower's (1994) widely accepted estimate was based on uncorrected pairwise distances. The application of this "standard rate" using uncorrected distances has been common practice in the entomological literature (e.g., Bernhard et al. 2005; Baker et al. 2008; Canfield et al. 2008) but may lead to incorrect conclusions, particularly when estimating older divergences. In the case of Aegean tenebrionids, if we had applied a 2.3% divergence rate without correcting for multiple hits, estimated ages of the six east/west nodes would be 3.6–5.4 Mya and support a scenario of post-Messinian diversification for these lineages.

Conversely, using the geological age of the mid-Aegean trench to estimate the substitution rate of the *cox1* + *rrnL* data set without using a gamma correction resulted in rates as low as 1% or 1.2% (fig. 2). However, when accounting for rate heterogeneity among sites, either assuming a strict or a relaxed clock, the estimates were much higher (2.23% with MrBayes, 2.39% with BEAST), demonstrating that rates are greatly underestimated if they are derived from uncorrected pairwise distances (Yang 1996; Arbogast et al. 2002). This was also evident from the compilation of literature values of studies using uncorrected versus model-based methods (including a gamma distribution to account for rate heterogeneity among sites) to estimate substitution rates, which showed that the values plateau at 1.5% versus 2.1%, respectively (fig. 3).

The choice of sequence evolution models requires careful consideration. Recent advances in model-based phylogenetics have suggested that it is often preferable to apply partition-specific rate models instead of using a single gamma distribution to describe the heterogeneity of the substitution process across multiple-gene regions and codon positions (Sullivan and Joyce 2005). Partitioned models have been shown to improve likelihood scores considerably (Castoe et al. 2004; Brandley et al. 2005), while having a great effect on branch lengths (Marshall et al. 2006) and divergence time estimates (Yang and Yoder 2003). In this study, we found the partitioning to cause an increase in inferred rates or ages by up to 12–14% (fig. 2 and table 5), with the deeper nodes affected proportionally more than the most recent nodes and a parallel increase in marginal likelihood as assessed by the harmonic mean estimator (table 4). This is in agreement with other recent studies showing an effect of the partitioning scheme on estimated divergence times (Poux et al. 2008; Torres-Carvajal and de Queiroz 2009), particularly in cases where a single calibration point is used or when an externally estimated standard molecular clock is applied (Marshall et al. 2006; Papadopoulou, Jones, et al. 2009). However, the different criteria for selecting the preferred partitioning scheme using Bayes factors (favoring the P3 or the most parameter-rich partitioning scheme P4) resulted in very similar substitution rates (2.68% vs. 2.69%), indicating that overparameterization has little impact on the estimates.

**Table 5.** Estimated Mean Ages ± 1 SD for Each of the East–West Nodes When Applying the Standard 2.3% Divergence $My^{-1}$ Rate on the mtDNA Data set Either Unpartitioned (P0) or Under Four Alternative Partitioning Schemes (P1–P4) or on *cox1* and *rrnL* Separately.

| | mtDNAP0[a] | P1[b] | P2[c] | P3[d] | P4[e] | *cox1* (1)[a] | *cox1* (2)[c] | *cox1* (3)[f] | *rrnL* |
|---|---|---|---|---|---|---|---|---|---|
| EW1 | 10.9 ± 1.37 | 10.97 ± 1.47 | 11.77 ± 1.5 | 11.8 ± 1.58 | 11.79 ± 1.58 | 14.81 ± 2.25 | 17 ± 2.7 | 16.81 ± 2.73 | 4.96 ± 1.31 |
| EW2 | 8.67 ± 0.87 | 9.61 ± 1.07 | 9.47 ± 1.01 | 9.76 ± 1.07 | 9.87 ± 1.08 | 12.32 ± 1.46 | 12.9 ± 1.61 | 13.09 ± 1.66 | 4.34 ± 1 |
| EW3 | 4.78 ± 0.78 | 4.85 ± 0.83 | 4.85 ± 0.85 | 4.92 ± 0.87 | 4.91 ± 0.86 | 7.09 ± 1.3 | 7.45 ± 1.53 | 7.46 ± 1.55 | 2.12 ± 0.93 |
| EW4 | 9.67 ± 1.65 | 9.87 ± 1.72 | 9.81 ± 1.78 | 10.03 ± 1.84 | 10.01 ± 1.82 | 13.38 ± 2.33 | 13.98 ± 2.69 | 13.96 ± 2.77 | 3.03 ± 1.25 |
| EW5 | 11.64 ± 1.35 | 12.34 ± 1.51 | 12.38 ± 1.5 | 12.47 ± 1.54 | 12.42 ± 1.54 | 16.41 ± 2.03 | 16.88 ± 2.13 | 16.68 ± 2.15 | 4.34 ± 1.15 |
| EW6 | 7.26 ± 0.8 | 7.38 ± 0.87 | 7.42 ± 0.85 | 7.65 ± 0.91 | 7.63 ± 0.9 | 9.46 ± 1.24 | 10.18 ± 1.41 | 10.17 ± 1.43 | 3.49 ± 0.77 |
| EW7 | 12.22 ± 1.96 | 13.68 ± 2.21 | 12.78 ± 2.19 | 13.64 ± 2.38 | 13.98 ± 2.41 | 17.55 ± 3.07 | 17.59 ± 3.4 | 18.25 ± 3.56 | 4.15 ± 1.41 |
| EW8 | 10.73 ± 1.33 | 12.04 ± 1.59 | 11.52 ± 1.52 | 11.9 ± 1.59 | 12.15 ± 1.62 | 15.57 ± 2.16 | 15.53 ± 2.35 | 15.83 ± 2.38 | 4.37 ± 1.19 |

[a] Unpartitioned.
[b] *cox1* versus *rrnL*.
[c] 3rd codons versus all other sites.
[d] 1st and 2nd positions versus 3rd positions versus *rrnL*.
[e] 1st versus 2nd versus 3rd versus *rrnL*.
[f] Each codon position separately.

## Time Dependency of Molecular Rate Estimates: Phylogenetic Versus Coalescent Dating

Brower's (1994) estimate was based on relatively recent calibration points (300 years–3.25 My) and assumed a linear relationship of sequence divergence with time over this period. This ignores the much higher apparent rate of divergence in recently split lineages (Ho et al. 2005; Ho and Larson 2006) which is possibly due to the fact that (slightly) deleterious mutations persist as transient polymorphisms in large populations, before they are lost at phylogenetic scales (Penny 2005). Even if the exact timescale affected by these higher rates and the exponential distribution describing this effect has been debated (Emerson 2007; Ho et al. 2007), the J-shaped curve of inferred rates in the literature survey fits an exponential function (fig. 3) and is consistent with an increased intraspecific rate. However, there is uncertainty with these data because a recent calibration point is needed when using population-level data to estimate mutation rates (Ho et al. 2008), which is rarely available. Consequently, the upturn in this curve is largely due to a small number of attempts to calibrate an intraspecific rate for insect taxa (Clarke et al. 2001; Gratton et al. 2008), which resulted in very high mtDNA divergence estimates (5.7% and 19.2% $My^{-1}$, respectively). More extensive studies on mammals (including human populations) and birds where demographic data are available confirm this exponential relationship, in broad agreement with the extrapolations of our figure 3. Yet, studies in insects continue to apply the standard phylogenetic rate of 2.3% at the population level where intraspecific calibration points are not available (Pfeiler et al. 2007; Anducho-Reyes et al. 2008; Avtzis et al. 2008; Leschen et al. 2008; McLean et al. 2008; Meng et al. 2008) and therefore risk to overestimate the inferred evolutionary ages.

Likewise, given these great differences between genealogical and phylogenetic rates, it appears critical not to mix intraspecific and interspecific sequence data when estimating lineage ages. Here, we applied an explicit procedure for removing intraspecific variation using the GMYC model, which separates independently coalescing entities from interspecific divergences based on the branching rates that are described by different equations in population-level and phylogenetic data. This mixed model was strongly favored over the null model of a uniform branching process, indicating that indeed such coalescent groups exist in this data set and can be identified by the threshold value that defines the ML point for the transition in branching rates. The GMYC model therefore was used here to reduce the phylogenetic tree to those portions that are expected to exhibit the long-term substitution rate, thereby eliminating fast-evolving population level variation that might have resulted in an overall higher rate estimate.

## Caveats of Biogeographic Calibrations

The dense sampling across a putative biogeographic barrier also addresses the problem of a priori species delimitation and selection of target groups suitable for clock calibrations. Clock calibrations using biogeographical data, including those used by Brower (1994), are usually assessed based on population sampling around a "known" boundary, rather than detected in a broad survey of populations, and the divergence on either side of a given barrier is automatically ascribed to the geological events that led to the formation of the barrier. However, lineage divergence and barrier formation (and its timing) may be uncorrelated, even where multiple codistributed taxon pairs are found to be subdivided across a given barrier (Heads 2005). For example, the final rise of the Isthmus of Panama at 2.7–3.5 Mya has been widely employed for molecular clock calibrations in marine taxa, but their ancestors may have diverged well before the closure of the Isthmus (Knowlton and Weigt 1998; Marko 2002) resulting in overestimated substitution rates. Here, instead of assuming a priori that all east/west splits can be attributed to a single biogeographic event, we sampled populations comprehensively across a wide geographic area without prior assumption of a biogeographic boundary and, once a major genetic break was found consistent with known geological features (the mid-Aegean trench), only those taxa showing broad temporal congruence across this border were retained for the calibration, using a relative dating approach (Loader et al. 2007).

Another important consideration when using biogeographic separations to calibrate molecular clocks is the fact that gene divergence generally predates population divergence due to ancestral polymorphism (Edwards and Beerli 2000), which may greatly affect the divergence time estimates when looking at recently separated populations. However, the amount of error will be proportionally smaller when divergence time is long relatively to the effective population size of the ancestral population (Edwards and Beerli 2000; Hurt et al. 2009). In the example of the Isthmus of Panama, recent coalescent-based reanalyses of multiple "geminate" species data sets found that a large proportion of the observed variation in genetic distances among taxa can be explained without invoking scenarios of nonsimultaneous divergence if ancestral polymorphism and among-taxon differences in demographic history are taken into account (Hickerson et al. 2006; Hurt et al. 2009). Nonetheless, applying a similar approach on the Aegean tenebrionid data set was not considered appropriate as this data set concerns more ancient divergences, whereas each of the eastern and western clades are composed of multiple independently coalescing groups confined to sets of adjacent islands. Lineage sorting therefore might affect the relationships at the level of sets of coalescence groups within each of the eastern and western clades but is unlikely to go as far back as the east/west splits themselves. Hence, the assumptions of current coalescence-based approaches for time calibrations that require an estimate of the effective population size to be taken into account (as implemented, e.g., in the msBayes software; Hickerson et al. 2007) are not fulfilled. Moreover, our calibration was performed on all nodes simultaneously, using an uncorrelated relaxed-clock method, which permitted us to estimate a mean rate in the face of stochastic variation in coalescent times and rate heterogeneity among lineages.

We also intended to minimize the confounding effects of postseparation gene flow by selecting focal taxa that are predicted to exhibit very low dispersal propensity given their morphological traits and their habitat association (Papadopoulou, Anastasiou, et al. 2009). For example, the geophilic lineages used here coexist on the same islands with "psammophilic" tenebrionids confined to ephemeral sandy habitats (beaches, sand dunes) and winged lineages. In these groups, dispersal becomes a prerequisite for lineage persistence and consequently these taxa show much weaker geographic structure in the same island system, (Papadopoulou, Anastasiou, et al. 2009), so they are not suitable for clock calibrations based on vicariance patterns. However, despite the careful choice of study taxa, our results illustrate the difficulty of correlating a geologically dated event with the timing of the lineage split. Ocean barriers that separated eastern and western lineages may have broken down during the Messinian drying events, and therefore, the six older contemporaneous east/west splits might be attributable to a more recent event along the same biogeographic boundary. Under this scenario, the original geological separation that lasted at least 3 My (9 to 5.96 Mya) would have left no signature on the diversification of these lineages, that is, all lineages would have acquired wide distribution in the Messinian before again being subjected to vicariant separation, which seems unlikely. This scenario would also leave the younger EW3 node unexplained which now can be associated with the post-Messinian split. Attributing the six older divergence events to the original formation of the trench therefore is considered the most parsimonious explanation for the observed patterns.

## Calibrating the Insect Molecular Clock

The final estimate for the mean mtDNA divergence rate in the Aegean tenebrionids of 2.39% $My^{-1}$ under a relaxed clock and applying a GTR+Γ+I or 2.69% under the preferred partitioning scheme (fig. 2) was remarkably similar to Brower's (1994) estimate and might give credibility to this widely applied rate. However, the current analysis shows that we should guard ourselves against accepting this number as a "universal" clock rate. The way these two figures were arrived at could not be more different, as they were obtained from very recent (mostly $< 1$ My) versus older ($10.5 \pm 1.5$ My) calibration ages, uncorrected versus corrected substitution rates, and separate versus simultaneous estimates from multiple calibrations. In addition, Brower's (1994) estimations were based on a diversity of data sets from *cox1*, *cox2*, *rrnS*, *rrnL*, and restriction sites that are not easily comparable. Our estimates for Aegean tenebrionids are a composite of *cox1* and slowly evolving *rrnL* genes (for a rate of 3.54% and 1.06%, respectively), and therefore the close similarity to the standard rate reflects an average of two quite divergent estimates. Finally, Brower's (1994) estimate refers to a path across the tree (i.e., % divergence between terminal taxa per time unit), whereas BEAST estimates a mean rate along the root-to-tip axis (expected number of substitutions per site per time unit). Under certain conditions, these two ways to assess rates can produce different results, particularly if there is not rate constancy across the tree. Therefore, it would be preferable to express our rate estimates as substitutions/site/My rather than % divergence/My, although we maintained the latter here (and converted all numbers accordingly) for consistency with the existing literature.

Even if Brower's (1994) calibration was based on a range of different genes, as *cox1* is widely used in insect phylogenetics and in many studies is the only marker available, the 2.3% rate has been often applied to *cox1*-only data sets. Furthermore, since Farrell (2001) and Quek et al. (2004) both reported a rate of 1.5% for *cox1*, several studies (Dick et al. 2004; Kandul et al. 2004; Zhang et al. 2005; Steiner et al. 2006; Bell et al. 2007; Aoki et al. 2008; Canfield et al. 2008; Kiyoshi 2008; Leschen et al. 2008; Wirta et al. 2008; Kawakita and Kato 2009) employed this lower rate for *cox1* data sets. Here, we found the substitution rate of *cox1* to be more than twice as fast as the estimates of Farrell (2001) and Quek et al. (2004). Their low estimates can easily be explained as an artifact of using uncorrected pairwise distances, whereas their calibration points were comparatively ancient (up to 20 Mya). Higher rates for

*cox1* have been estimated in various insect lineages by Pons and Vogler (2005) (3.34%; Coleoptera: Cicindelidae), Shapiro et al. (2006) (3–4%; Orthoptera: Tettigoniidae), Kiyoshi and Sota (2006) (3.1%; Odonata: Gomphidae), Percy et al. (2004) (2.35–3.15%; Hemiptera: Psyllidae), and Nazari and Sperling (2007) (2.3–3.1%; Lepidoptera: Papilionidae). Nonetheless, *cox1* appears to be among the most slowly evolving protein-coding mitochondrial genes (Crozier RH and Crozier YC 1993), which is corroborated by recent studies (e.g., Pons and Vogler 2005: Cytochrome b (*cob*) 4.22% vs. *cox1* 3.34%). Therefore, our estimates of the mtDNA clock would likely be higher using other protein-coding genes. In contrast, the mitochondrial rRNA genes generally diverge more slowly (Trewick and Wallis 2001), in agreement with our estimate for *rrnL* (1.08%) which is still higher than the rates proposed by Gómez-Zurita et al. (2000) (0.45%, Chrysomelidae), Pons and Vogler (2005) (0.76%, Cicindelidae) and it falls into the range reported by Percy et al. (2004) (0.95–1.9%, Psyllidae). Hence, there are consistent, large differences in rates among mitochondrial genes and therefore standard rates to date phylogenies in the absence of other independent evidence need to be based on gene-specific calibrations.

Regarding the nuclear genes, the mean rate obtained for the intron region of Mp20 (3.68%) is close to the numbers reported for synonymous substitutions in Drosophila (3% by Rowan and Hunt 1991; 3.08% by Li 1997) and for a "numt" pseudogene in *Cicindela* (3.33%; Pons and Vogler 2005). Moreover, it has been proposed that the average rate of neutral single-copy nuclear DNA in insects is similar to the mtDNA rate (Caccone et al. 1988; Sharp and Li 1989), which is consistent with our results, although our estimate for the intron rate remains preliminary, as the SD is large due to the small size of the intron and the stochastic nature of the substitution process. However, the fact that this rate is similar to those expected from independent studies provides confidence in the calibration based on the formation of the mid-Aegean trench and therefore also supports the mtDNA rates established here.

Overall, the substitution rates found here for the Aegean tenebrionids appear slightly higher than most previous estimates for insects, which could be partially due to the particular mode of diversification of these island lineages. High speciation rates have been associated with elevated rates of molecular evolution (Barraclough and Savolainen 2001; Webster et al. 2003), whereas lineages with small effective populations sizes are also predicted to have increased substitution rates due to the greater rate of fixation of nearly neutral alleles (Ohta 1987; Woolfit 2009). However, the hypothesis that island radiations can speed up the molecular clock has been tested explicitly in a range of different data sets and has found no support (Bromham and Woolfit 2004).

## Conclusion

MtDNA substitution rates are frequently the only source of information to date historical scenarios. The desire for a standard rate of change is therefore not surprising but the uncritical use of these rates to calibrate phylogenetic trees is fraught with errors. Here, we established the methodological issues that impact rate estimates, with the correction for rate heterogeneity among sites and the choice of genes having the greatest effect. However, despite great differences in reported rate estimates, the discrepancies can be largely explained by differences in methodology (fig. 3) without invoking large differences in the actual mtDNA substitution rate. More data sets of this kind that reduce stochastic effects by using multiple independent calibration points are needed to assess the subtle changes in rates among lineages with greater precision. This will establish whether or not the comparatively high rates found in the Aegean tenebrionids are unique to this group or reflect generally an underestimate of the standard rate for *cox1*.

## Supplementary Material

Supplementary tables S1–S9 and figures S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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## References

Andersen NM, Cheng L, Damgaard J, Sperling FAH. 2000. Mitochondrial DNA sequence variation and phylogeography of oceanic insects (Hemiptera: Gerridae: Halobates spp.). *Mar Biol.* 136(3):421–430.

Anducho-Reyes MA, Cognato AI, Hayes JL, Zuniga G. 2008. Phylogeography of the bark beetle Dendroctonus mexicanus Hopkins (Coleoptera: Curculionidae: Scolytinae). *Mol Phylogenet Evol.* 49(3):930–940.

Aoki K, Kato M, Murakami N. 2008. Glacial bottleneck and postglacial recolonization of a seed parasitic weevil, Curculio hilgendorfi, inferred from mitochondrial DNA variation. *Mol Ecol.* 17(14):3276–3289.

Arbogast BS, Edwards SV, Wakeley J, Beerli P, Slowinski JB. 2002. Estimating divergence times from molecular data on phylogenetic and population genetic timescales. *Annu Rev Ecol Syst.* 33:707–740.

Avtzis DN, Arthofer W, Stauffer C. 2008. Sympatric occurrence of diverged mtDNA lineages of Pityogenes chalcographus (Coleoptera, Scolytinae) in Europe. *Biol J Linn Soc Lond.* 94(2): 331–340.

Baker CH, Graham GC, Scott KD, Cameron SL, Yeates DK, Merritt DJ. 2008. Distribution and phylogenetic relationships of Australian glow-worms Arachnocampa (Diptera, Keroplatidae). *Mol Phylogenet Evol.* 48(2):506–514.

Barraclough TG, Savolainen V. 2001. Evolutionary rates and species diversity in flowering plants. *Evolution.* 55:677–683.

Bell KL, Moritz C, Moussalli A, Yeates DK. 2007. Comparative phylogeography and speciation of dung beetles from the Australian Wet Tropics rainforest. *Mol Ecol.* 16(23):4984–4998.
Bernhard D, Fritzsch G, Glockner P, Wurst C. 2005. Molecular insights into speciation in the Agrilus viridis-complex and the genus Trachys (Coleoptera: Buprestidae). *Eur J Entomol.* 102(4): 599–605.
Brandley MC, Schmitz A, Reeder TW. 2005. Partitioned Bayesian analyses, partition choice, and the phylogenetic relationships of scincid lizards. *Syst Biol.* 54(3):373–390.
Bromham L, Woolfit M. 2004. Explosive radiations and the reliability of molecular clocks: island endemic radiations as a test case. *Syst Biol.* 53(5):758–766.
Brower AVZ. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA Evolution. *Proc Natl Acad Sci U S A.* 91:6491–6495.
Caccone A, Amato GD, Powell JR. 1988. Rates and patterns of scnDNA and mtDNA divergence within the *Drosophila melanogaster* subgroup. *Genetics* 118(4):671–683.
Canfield MR, Greene E, Moreau CS, Chen N, Pierce NE. 2008. Exploring phenotypic plasticity and biogeography in emerald moths: a phylogeny of the genus Nemoria (Lepidoptera: Geometridae). *Mol Phylogenet Evol.* 49(2):477–487.
Carstens BC, Knowles LL. 2007. Shifting distributions and speciation: species divergence during rapid climate change. *Mol Ecol.* 16(3): 619–627.
Castoe TA, Doan TM, Parkinson CL. 2004. Data partitions and complex models in Bayesian analysis: the phylogeny of Gymnophthalmid lizards. *Syst Biol.* 53(3):448–469.
Clarke TE, Levin DB, Kavanaugh DH, Reimchen TE. 2001. Rapid evolution in the Nebria gregaria group (Coleoptera: Carabidae) and the paleogeography of the Queen Charlotte Islands. *Evolution* 55(7):1408–1418.
Cognato AI, Vogler AP. 2001. Exploring data interaction and nucleotide alignment in a multiple gene analysis of *Ips* (Coleoptera: Scolytinae). *Syst Biol.* 50:758–780.
Creutzburg N. 1963. Paleogeographic evolution of Crete from Miocene till our days. *Cretan Ann.* 15–16:336–342.
Crozier RH, Crozier YC. 1993. The mitochondrial genome of the honeybee Apis mellifera: complete sequence and genome organization. *Genetics* 133(1):91–117.
Dermitzakis DM, Papanikolaou DJ. 1981. Paleogeography and geodynamics of the Aegean region during the Neogene. *Annales Géologique des Pays Hellénique.* 30:245–289.
Dick CW, Roubik DW, Gruber KF, Bermingham E. 2004. Long-distance gene flow and cross-Andean dispersal of lowland rainforest bees (Apidae: Euglossini) revealed by comparative mitochondrial DNA phylogeography. *Mol Ecol.* 13(12): 3775–3785.
Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. 2006. Relaxed phylogenetics and dating with confidence. *PLoS Biol.* 4(5): 699–710.
Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol.* 7:214
Edwards SV, Beerli P. 2000. Perspective: gene divergence, population divergence, and the variance in coalescence time in phylogeographic studies. *Evolution* 54(6):1839–1854.
Emerson BC. 2007. Alarm bells for the molecular clock? No support for Ho et al.'s model of time-dependent molecular rate estimates. *Syst Biol.* 56(2):337–345.
Farrell BD. 2001. Evolutionary assembly of the milkweed fauna: cytochrome oxidase I and the age of Tetraopes beetles. *Mol Phylogenet Evol.* 18(3):467–478.
Farris JS, Källersjö M, Kluge AG, Bult C. 1994. Testing significance of incongruence. *Cladistics* 10:315–319.
Fleischer RC, McIntosh CE, Tarr CL. 1998. Evolution on a volcanic conveyor belt: using phylogeographic reconstructions and K-Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates. *Mol Ecol.* 7(4):533–545.
Fontaneto D, Herniou EA, Boschetti C, Caprioli M, Melone G, Ricci C, Barraclough TG. 2007. Independently evolving species in asexual bdelloid rotifers. *PLoS Biol.* 5:914–921.
Gaunt MW, Miles MA. 2002. An insect molecular clock dates the origin of the insects and accords with palaeontological and biogeographic landmarks. *Mol Biol Evol.* 19(5):748–761.
Gómez-Zurita J, Juan C, Petitpierre E. 2000. The evolutionary history of the genus *Timarcha* (Coleoptera, Chrysomelidae) inferred from mitochondrial COII gene and partial 16S rDNA sequences. *Mol Phylogenet Evol.* 14:304–317.
Gratton P, Konopinski MK, Sbordoni V. 2008. Pleistocene evolutionary history of the Clouded Apollo (Parnassius mnemosyne): genetic signatures of climate cycles and a 'time-dependent' mitochondrial substitution rate. *Mol Ecol.* 17(19): 4248–4262.
Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol.* 52:696–704.
Heads M. 2005. Dating nodes on molecular phylogenies: a critique of molecular biogeography. *Cladistics* 21(1):62–78.
Hickerson MJ, Gilchrist MA, Takebayashi N. 2003. Calibrating a molecular clock from phylogeographic data: moments and likelihood estimators. *Evolution* 57(10):2216–2225.
Hickerson MJ, Stahl E, Takebayashi N. 2007. msBayes: pipeline for testing comparative phylogeographic histories using hierarchical approximate Bayesian computation. *BMC Bioinformatics.* 8:268.
Hickerson MJ, Stahl EA, Lessios HA. 2006. Test for simultaneous divergence using approximate Bayesian computation. *Evolution* 60(12):2435–2453.
Ho SYW. 2009. An examination of phylogenetic models of substitution rate variation among lineages. *Biol Lett.* 5(3):421–424
Ho SYW, Larson G. 2006. Molecular clocks: when times are a-changin'. *Trends Genet.* 22(2):79–83.
Ho SYW, Phillips MJ, Cooper A, Drummond AJ. 2005. Time dependency of molecular rate estimates and systematic over-estimation of recent divergence times. *Mol Biol Evol.* 22(7): 1561–1568.
Ho SYW, Saarma U, Barnett R, Haile J, Shapiro B. 2008. The effect of inappropriate calibration: three case studies in molecular ecology. *PLoS One.* 3(2):e1615.
Ho SYW, Shapiro B, Phillips MJ, Cooper A, Drummond AJ. 2007. Evidence for time dependency of molecular rate estimates. *Syst Biol* 56:515–522.
Hurt C, Anker A, Knowlton N. 2009. A multilocus test of simultaneous divergence across the Isthmus of Panama using snapping shrimp in the genus *Alpheus*. *Evolution* 63(2):514–530.
Kandul NP, Lukhtanov VA, Dantchenko AV, Coleman JWS, Sekercioglu CH, Haig D, Pierce NE. 2004. Phylogeny of Agrodiaetus Hubner 1822 (Lepidoptera: Lycaenidae) inferred from mtDNA sequences of COI and COII and nuclear sequences of EF1-alpha: karyotype diversification and species radiation. *Syst Biol* 53(2):278–298.
Kass RE, Raftery AE. 1995. Bayes factors. *J Am Stat Assoc.* 90(430): 773–795.
Katoh K, Kuma K, Toh H, Miyata T. 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res.* 33:511–518.
Katoh K, Toh H. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform.* 9(4):286–298.
Kawakita A, Kato M. 2009. Repeated independent evolution of obligate pollination mutualism in the Phyllantheae-Epicephala association. *Proc R Soc Lond B Biol Sci.* 276(1656):417–426.

Kiyoshi T. 2008. Differentiation of golden-ringed dragonfly Anotogaster sieboldii (Selys, 1854) (Cordulegastridae: Odonata) in the insular East Asia revealed by the mitochondrial gene genealogy with taxonomic implications. *J Zool Syst Evol Res.* 46(2):105–109.

Kiyoshi T, Sota T. 2006. Differentiation of the dragonfly genus Davidius (Odonata: Gomphidae) in Japan inferred from mitochondrial and nuclear gene genealogies. *Zool Sci.* 23(1):1–8.

Knowlton N, Weigt LA. 1998. New dates and new rates for divergence across the Isthmus of Panama. *Proc R Soc Lond B Biol Sci.* 265(1412):2257–2263.

Krijgsman W, Hilgen FJ, Raffi I, Sierro FJ, Wilson DS. 1999. Chronology, causes and progression of the Messinian salinity crisis. *Nature* 400(6745):652–655.

Leschen RAB, Buckley TR, Harman HM, Shulmeister J. 2008. Determining the origin and age of the Westland beech (*Nothofagus*) gap, New Zealand, using fungus beetle genetics. *Mol Ecol.* 17(5):1256–1276.

Li WH. 1997. Molecular evolution. Sunderland (MA): Sinauer.

Loader SP, Pisani D, Cotton JA, Gower DJ, Day JJ, Wilkinson M. 2007. Relative time scales reveal multiple origins of parallel disjunct distributions of African caecilian amphibians. *Biol Lett.* 3: 505–508.

Luchetti A, Marini M, Mantovani B. 2005. Mitochondrial evolutionary rate and speciation in termites: data on European Reticulitermes taxa (Isoptera, Rhinotermitidae). *Insectes Soc.* 52(3):218–221.

Marko PB. 2002. Fossil calibration of molecular clocks and the divergence times of geminate species pairs separated by the Isthmus of Panama. *Mol Biol Evol.* 19(11):2005–2021.

Marshall DC, Simon C, Buckley TR. 2006. Accurate branch length estimation in partitioned Bayesian analyses requires accommodation of among-partition rate variation and attention to branch length priors. *Syst Biol.* 55(6):993–1003.

McLean AJ, Schmidt DJ, Hughes JM. 2008. Do lowland habitats represent barriers to dispersal for a rainforest mayfly, Bungona narilla, in South-East Queensland? *Mar Freshw Res.* 59(9):761–771.

Meng XF, Shi M, Chen XX. 2008. Population genetic structure of Chilo suppressalis (Walker) (Lepidoptera: Crambidae): strong subdivision in China inferred from microsatellite markers and mtDNA gene sequences. *Mol Ecol.* 17(12):2880–2897.

Miller KB, Bergsten J, Whiting MF. Forthcoming. 2009. Phylogeny and classification of the tribe Hydaticini (Coleoptera: Dytiscidae): partition choice for Bayesian analysis with multiple nuclear and mitochondrial protein-coding genes. *Zool Scr.* 38(6):591–615

Monaghan MT, Inward DJG, Hunt T, Vogler AP. 2007. A molecular phylogenetic analysis of the Scarabaeinae (dung beetles). *Mol Phylogenet Evol.* 45:674–692.

Nazari V, Sperling FAH. 2007. Mitochondrial DNA divergence and phylogeography in western Palaearctic Parnassiinae (Lepidoptera: Papilionidae): how many species are there? *Insect Syst Evol.* 38(2):121–138.

Nylander JAA. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Ohta T. 1987. Very slightly deleterious mutations and the molecular clock. *J Mol Evol.* 26(1–2):1–6.

Pagel M, Meade A. 2004. A phylogenetic mixture model for detecting pattern-heterogeneity in gene sequence or character-state data. *Syst Biol.* 53(4):571–581.

Papadopoulou A, Anastasiou I, Keskin B, Vogler AP. 2009. Comparative phylogeography of tenebrionid beetles in the Aegean archipelago: the effect of dispersal ability and habitat preference. *Mol Ecol.* 18:2503–2517.

Papadopoulou A, Jones AG, Hammond PM, Vogler AP. 2009. DNA taxonomy and phylogeography of beetles of the Falkland Islands (Islas Malvinas). *Mol Phylogenet Evol.* 53(3):935–947.

Penny D. 2005. Evolutionary biology—relativity for molecular clocks. *Nature* 436(7048):183–184.

Percy DM, Page RDM, Cronk QCB. 2004. Plant-insect interactions: double-dating associated insect and plant lineages reveals asynchronous radiations. *Syst Biol.* 53(1):120–127.

Pfeiler E, Erez T, Hurtado LA, Markow TA. 2007. Genetic differentiation and demographic history in Drosophila pachea from the Sonoran Desert. *Hereditas* 144(2):63–74.

Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD, Vogler AP. 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Syst Biol.* 55:595–609.

Pons J, Barraclough TG, Theodorides K, Cardoso A, Vogler AP. 2004. Using exon and intron sequences of the gene Mp20 to resolve basal relationships in *Cicindela* (Coleoptera: Cicindelidae). *Syst Biol.* 53:554–570.

Pons J, Vogler AP. 2005. Complex pattern of coalescence and fast evolution of a mitochondrial rRNA pseudogene in a recent radiation of tiger beetles. *Mol Biol Evol.* 22:991–1000.

Poux C, Madsen O, Glos J, de Jong WW, Vences M. 2008. Molecular phylogeny and divergence times of Malagasy tenrecs: influence of data partitioning and taxon sampling on dating analyses. *BMC Evol Biol.* 8:102

Pruser F, Mossakowski D. 1998. Low substitution rates in mitochondrial DNA in Mediterranean carabid beetles. *Insect Mol Biol.* 7(2):121–128.

Quek SP, Davies SJ, Itino T, Pierce NE. 2004. Codiversification in an ant-plant mutualism: stem texture and the evolution of host use in Crematogaster (Formicidae: Myrmicinae) inhabitants of Macaranga (Euphorbiaceae). *Evolution* 58(3):554–570.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19(12): 1572–1574.

Rowan RG, Hunt JA. 1991. Rates of DNA change and phylogeny from the DNA-sequences of the alcohol-dehydrogenase for 5 closely related species of Hawaiian *Drosophila*. *Mol Biol Evol.* 8(1):49–70.

Sanderson MJ. 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Mol Biol Evol* 19:101–109.

Sanderson MJ. 2003. r8s: inferring absolute rates of molecular evolution, divergence times in the absence of a molecular clock. *Bioinformatics* 19:301–302.

Shapiro LH, Strazanac JS, Roderick GK. 2006. Molecular phylogeny of Banza (Orthoptera: Tettigoniidae), the endemic katydids of the Hawaiian Archipelago. *Mol Phylogenet Evol.* 41(1):53–63.

Sharp PM, Li WH. 1989. On the rate of DNA-sequence evolution in *Drosophila*. *J Mol Evol.* 28(5):398–402.

Shimodaira H, Hasegawa M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol Biol Evol.* 16:1114–1116.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann Entomol Soc Am.* 87:651–701.

Sperling FAH, Spence JR, Andersen NM. 1997. Mitochondrial DNA, allozymes, morphology, and hybrid compatibility in Limnoporus water striders (Heteroptera: Gerridae): do they all track species phylogenies? *Ann Entomol Soc Am.* 90(4):401–415.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22(21):2688–2690.

Stamatakis A, Hoover P, Rougemont J. 2008. A rapid bootstrap algorithm for the RAxML web servers. *Syst Biol.* 57(5):758–771.

Steiner FM, Schlick-Steiner BC, Konrad H, Linksvayer TA, Quek SP, Christian E, Stauffer C, Buschinger A. 2006. Phylogeny and

evolutionary history of queen polymorphic Myrmecina ants (Hymenoptera: Formicidae). *Eur J Entomol.* 103(3):619–626.
Sullivan J, Joyce P. 2005. Model selection in phylogenetics. *Ann Rev Ecology Evol Syst.* 36:445–466.
Swofford DL. 2002. PAUP*: phylogenetic analysis using parsimony. Version 4.0b. Sunderland (MA): Sinauer Associates.
Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol.* 24(8):1596–1599.
Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673–4680.
Thorne JL, Kishino H. 2002. Divergence time and evolutionary rate estimation with multilocus data. *Syst Biol.* 51(5):689–702.
Thornton I. 2007. Island colonization: the origin and development of Island communities. New York: Cambridge University Press.
Torres-Carvajal O, de Queiroz K. 2009. Phylogeny of hoplocercine lizards (Squamata: Iguania) with estimates of relative divergence times. *Mol Phylogenet Evol.* 50(1):31–43.
Trewick SA, Wallis GP. 2001. Bridging the "beech-gap": New Zealand invertebrate phylogeography implicates Pleistocene glaciation and Pliocene isolation. *Evolution* 55(11):2170–2180.
Webster AJ, Payne RJH, Pagel M. 2003. Molecular phylogenies link rates of evolution and speciation. *Science* 301(5632):478–478.
Wheeler WC, Hayashi CY. 1998. The phylogeny of the extant chelicerate orders. *Cladistics* 14:173–192.
Wirta H, Orsini L, Hanski I. 2008. An old adaptive radiation of forest dung beetles in Madagascar. *Mol Phylogenet Evol.* 47(3):1076–1089.
Woolfit M. 2009. Effective population size and the rate and pattern of nucleotide substitutions. *Biol Lett.* 5(3):417–420.
Yang Z. 2004. A heuristic rate smoothing procedure for maximum likelihood estimation of species divergence times. *Acta Zool Sinica.* 50:645–656.
Yang ZH. 1996. Maximum-likelihood models for combined analyses of multiple sequence data. *J Mol Evol.* 42(5):587–596.
Yang ZH, Yoder AD. 2003. Comparison of likelihood and Bayesian methods for estimating divergence times using multiple gene loci and calibration points, with application to a radiation of cute-looking mouse lemur species. *Syst Biol.* 52(5):705–716.
Zhang AB, Kubota K, Takami Y, Kim JL, Kim JK, Sota T. 2005. Species status and phylogeography of two closely related Coptolabrus species (Coleoptera: Carabidae) in South Korea inferred from mitochondrial and nuclear gene sequences. *Mol Ecol.* 14(12): 3823–3841.