# ORIGINAL ARTICLE

# QUANTIFYING THE PLEISTOCENE HISTORY OF THE OAK GALL PARASITOID *CECIDOSTIBA FUNGOSA* USING TWENTY INTRON LOCI

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The longitudinal spread of temperate organisms into refugial populations in Southern Europe is generally assumed to predate the last interglacial. However few studies have attempted to quantify this process in nonmodel organisms using explicit models and multilocus data. We used sequence data for 20 intron-spanning loci (12 kb per individual) to resolve the history of refugial populations of a widespread western Palaearctic oak gall parasitoid *Cecidostiba fungosa* (Pteromalidae). Using maximum likelihood and Bayesian methods we assess alternative population tree topologies and estimate divergence times and ancestral population sizes under a model of divergence between three refugia (Middle East, Balkans and Iberia). Both methods support an "Out of the East" history for *C. fungosa*, matching the pattern previously inferred for their gallwasp hosts. However, coalescent-based estimates of the ages of population divides are much more recent (coinciding with the Eemian interglacial) than nodal ages of single gene trees for *C. fungosa* and other species. We also find that increasing the sample size from one haploid sequence per refugial population to three only marginally improves parameter estimates. Our results suggest that there is significant information in the minimal samples currently analyzable with maximum likelihood methods, and that similar methods could be applied to multiple species to test alternative models of assemblage evolution.

**KEY WORDS:** Ancestral population size, coalescent theory, parasitoid assemblages, population divergence times, statistical phylogeography.

Many western palaearctic taxa have their current centers of genetic diversity to the east of Europe, suggesting that refugial populations around the Mediterranean basin are ultimately derived from a more eastern source (Din et al. 1996; Rokas et al. 2003; Juste et al. 2004; Michaux et al. 2004; Culling et al. 2006; Koch et al. 2006; Challis et al. 2007; Stone et al. 2007). Westwards dispersal of such taxa into southern European refugia is often thought to have occurred in the early Pleistocene, if not before (Taberlet et al. 1998; Rokas et al. 2003; Juste et al. 2004; Culling et al. 2006; Challis et al. 2007) and of necessity must predate the well-documented latitudinal range shifts associated with the

last ice age (Taberlet et al. 1998; Hewitt 1999) by at least one glacial cycle. However, the few studies that have attempted to estimate the age of this older longitudinal dispersal are largely qualitative, being based on a small set of (primarily mitochondrial) gene trees (e.g., Taberlet et al. 1998; Hewitt 1999; Nichols 2001; Rokas et al. 2003; Juste et al. 2004; Culling et al. 2006; Challis et al. 2007). It has been noted that species differ considerably in their mitochondrial divergence between refugia and this has been attributed to species-specific responses to Pleistocene climate cycles (Taberlet et al. 1998). However, an obvious alternative explanation for the observed lack of interspecific temporal

congruence is that mitochondrial gene trees are dominated by incomplete lineage sorting, the extent of which may be large in general and/or different between species (Nichols 2001).

Because polymorphism within ancestral populations must originate before daughter populations diverge, branches of gene trees are necessarily longer than those of population trees and a naïve interpretation of node ages may severely overestimate population divergence (Pamilo and Nei 1988; Maddison 1997). Similarly, gene tree topologies may be incongruent with the order of population divergence (Tajima 1983; Pamilo and Nei 1988; Rosenberg 2002). Because the magnitude of both these effects depends on the size and stability of the ancestral populations (Tajima 1983; Maddison 1997; Nichols 2001), they are likely to be exaggerated when resolving the origins of—and relationships among—refugial populations, which are stable by their very nature (Hewitt 1999). Thus assessing the generality of an "Out of the East" pattern ideally requires replication both at the level of species and loci.

Assemblages of parasitoids associated with oak cynipid galls offer unmatched replication at the species level. In the Western Palaearctic, an estimated 120 species of chalcidoid wasps are obligate natural enemies of the inhabitants of oak cynipid galls (Csóka et al. 2005; Hayward and Stone 2005). Phylogeographic studies on Western Palaearctic oak gallwasps show their populations to be divided into three major refugial areas: the Iberian Peninsula in the west, Central Europe and the Balkans in the centre, and Asia Minor and Iran in the east (Rokas et al. 2001, 2003; Stone et al. 2001, 2008; Challis et al. 2007), broadly paralleling patterns seen in oak phylogeography (Dumolin-Lapegue et al. 1997). In the gallwasps, allele frequency data for multiple nuclear markers support the conclusion that there has been very little subsequent gene flow between these regions (Rokas et al. 2001, 2003; Stone et al. 2001, 2008; Challis et al. 2007). Oak gallwasps are thought to have diversified in regions to the east of Europe prior to the Pleistocene (Stone et al. 2009), and pre-Pleistocene or early Pleistocene westwards range expansion across Europe has been suggested by patterns of genetic variation in several widespread species (Rokas et al. 2001, 2003; Challis et al. 2007). An obvious question is whether gall-associated parasitoids have pursued their hosts from the east. At least two of them, the torymids *Megastigmus stigmatizans* and *M. dorsalis,* appear to have done so (Rokas et al. 2003; Hayward and Stone 2006; Nicholls et al. 2010). The challenge now is to reconstruct longitudinal colonization processes in the Western Palaearctic for a broader taxonomic spread of oak gall-associated parasitoids, to assess the generality of an "Out of the East" pattern, and to determine whether parasitoids dispersed over a similar timescale to their hosts, or after a delay—so allowing their hosts a measure of "enemy-free space" (Hayward and Stone 2006). One reason for caring which of these scenarios is true is that close phylo-
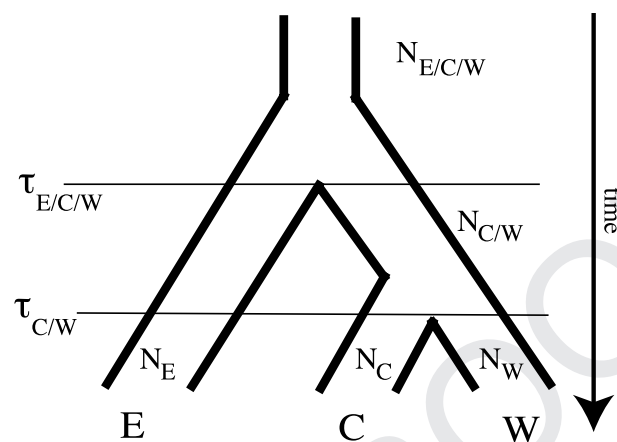


**Figure 1.** Model of successive population divergence between major Palearctic refugia from East to West: Asia Minor (E) Balkans and Central Europe (C), Iberia (W). With minimal sampling of one individual per population, topological probabilities of gene trees are determined by only two model parameters, the time between population divergences ($\tau_{E/C/W} - \tau_{C/W}$) and the effective sizes of the ancestral population during this time ($N_{C/W}$).

geographic concordance increases the potential for coevolution among community members, and such communities are inherently sensitive to disturbance by species gain (Stone and Sunnucks 1993; Schönrogge et al. 1996b, 1998) or loss (Lennartsson 2002; Pauw 2007).

Here, we use sequence data from 20 intronic loci to study the history of refugial populations in the pteromalid parasitoid *Cecidostiba fungosa*, a widespread species in oak gall communities (Askew 1961; Schönrogge et al. 1996a; Bailey et al. 2009). The three-refuge phylogeographic pattern of oak gallwasp communities allows us to compare two analytical methods—a maximum likelihood (ML) approach (Yang 2002), and an analogous, Bayesian approach (Rannala and Yang 2003). Both estimate ancestral population parameters (population sizes and divergence times) directly from patterns of polymorphism in sequence data (rather than from gene trees inferred for each locus) and assume a model of divergence between three populations (Fig. 1). The order of population divergence or the topology of the population tree can be viewed as an additional model parameter and the likelihoods in both methods can be used to compare statistical support for different topologies. We address the following, specific questions:

(1) Do data for C. fungosa support an "Out of the East" population history, such that refugial populations in the center and west of Europe are derived from a shared ancestral population in the center which in turn is derived from a common ancestral population further east (Fig. 1)?

(2) When did refugial populations split from each other, and how large were their ancestral populations?

(3) How different are multilocus estimates of population divergence times from gene divergence times (both nuclear and mitochondrial)?

A strategy of sampling many loci from a single individual per taxon, has been used extensively to study divergence between closely related species, in particular the Great Apes (Yang 2002; Jennings and Edwards 2005; Patterson et al. 2006). There are two reasons why such minimal sampling is of interest. First, going backwards in time, only lineages that persist into the ancestral species/population contribute to estimates of ancestral population parameters. Coalescent theory shows that samples taken from the same species or population quickly coalesce down to a small number of lineages (Griffiths 1981; Tavaré 1984; Norborg 1998) (Fig. 2). This means that even if divergence is relatively recent, that is, less than $N_e$ generations ago, the power gained by increasing within-population sampling levels off relatively rapidly. In contrast, each additional sampled locus provides an independent replicate of the coalescent process in the ancestral population irrespective of the divergence time (Wakeley 2004). So if the total cost of sampling is number of loci × number of sampled individuals, the optimal sampling scheme is one of few individuals sequenced at a large number of loci. Second, minimal sampling is currently the only sampling scheme for which a statistically optimal likelihood method allowing parameter estimation directly from site patterns exists (Yang 2002). In contrast, Bayesian approaches (Rannala and Yang 2003) or gene tree–species tree methods (Degnan and Salter 1995; Maddison and Knowles 2006; Liu and Pearl 2007; Degnan and Rosenberg 2009; Kubatko et al. 2009) have the advantage that they can deal with arbitrary sample sizes and numbers of populations. However, this comes at the potential cost of prior assumptions and/or difficulty in integration over topological uncertainty in the gene trees.

These issues are relevant in selecting an appropriate study design in systems in which there is a trade off in effort between sampling multiple individuals and generating data for multiple loci or species. Ability to obtain informative population parameters from small numbers of individuals is likely to be particularly important in comparative studies of communities, such as the oak gall system, in which some taxa are rare enough that increasing sample size is not an option. It is therefore useful to ask how much information about ancestral population parameters over phylogeographic timescales can be obtained with minimal sampling. To investigate the influence of sample size, we compared minimal sampling of a single individual per population with an extended sample of three individuals per population. We then use theoretical expectations for the number of surviving lineages given the estimated divergence history (Fig. 2) to consider the likely gain in power for larger sample sizes in our Discussion.

## *Methods*
### CHOICE OF LOCI

We obtained sequences for 20 newly developed intronic loci for *C. fungosa* (Table 1) and the closely related species *Caenacis lauta*, which was used as an outgroup in some analyses. These loci included 12 ribosomal protein genes (*RpL10ab, RpL13a, RpL15, RpL27a, RpL37, RpL37a, RpL39, RpS15, RpS18, RpS23, RpS4, RpS8*) and eight regulatory genes (*AntSesB, bellwether, nAcRbeta-64B, Rack1, Ran, sansfille, SUI, Tctp*) (for primer sequences and CG indentifiers see Table S1), all of which are thought to be single copy genes with no known paralogs in insects. Primer development and testing will be described in detail elsewhere (Lohse et al. unpubl. ms.). In short, primers were designed using  Q4 alignments of Hymenoptera EST data (Sharanowski et al. 2009) and insect sequences from public databases (NCBI). No or little polymorphism at a particular locus may arise either as a result of a low mutation rate (so limiting signal), or a recent coalescent event (and so important to demographic inference), or both. Excluding loci that are invariant in *C. fungosa* results in an upward bias in estimates of population divergence time. To avoid such bias, we used all nuclear loci available for *C. fungosa* (Lohse et al., unpubl. Ms.) and tested whether accounting for differences in mutation rate between loci influenced our estimates.
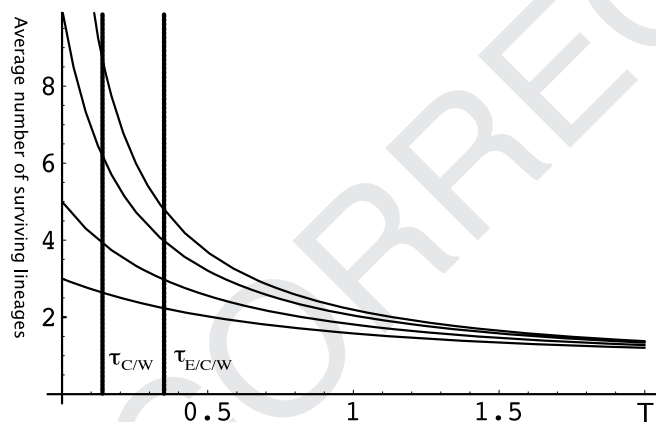


**Figure 2.** The expected mean number of lineages surviving coa-  Q2 lescence into an ancestral population (Tavare 1994, equation 5.5) plotted against divergence time (T) in coalescence units (2$N_e$ generations) for four different sampling sizes (from top to bottom, $n = 20, 10, 5, 3$). Because only surviving lineages contribute to the estimation of ancestral parameters and their number decreases rapidly, the expected gain in power from increasing sample size is limited even if divergence is relatively recent ($T < 0.5$). The solid lines show the divergence time estimates (scaled by twice the mean of population sizes $N_E$, $N_C$, and $N_W$) obtained for *C. fungosa* in this study (priors *a*).

**Table 1.** Summary statistics of nuclear loci in used in the analysis. Loci for which a larger sample of three individuals per population was obtained are shown in bold. Diversity within in the minimal single individual sample and divergence to *C. lauta* were calculated for introns ($\pi_{Intron}$, $K_{Intron}$) and synonymous exon sites ($\pi_S$, $K_S$) separately. Also shown are the number of introns (#In) and the total number of polymorphic sites (S) for the single individual samples and locus-specific mutation rate ($\mu$). The normalized product of $\mu$ and the total locus length can be taken as a measure of information content (Info). The last column (rec) gives the number of bases, which were excluded to trim each locus to the largest nonrecombining fragment according to the four-gamete tests.

| Locus | primers | #In | Length (bp) | | | Diversity | | | Divergence/mutation rate | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total | Intron | Exon | $\pi_s$ | $\pi_{Intron}$ | S | $K_s$ | $K_{Intron}$ | $\mu$ | Info | rec (bp) |
| **AntSesB** | 40fb, 40rb | 2 | 606 | 171 | 435 | 0.000 | 0.008 | 2 | 0.076 | 0.148 | 0.984 | 0.981 | 0 |
| bellwether | 33fb, 33rb | 1 | 549 | 214 | 335 | 0.000 | 0.003 | 2 | n/a | n/a | n/a | n/a | 0 |
| **nAcRbeta-64B** | 39f, 39r, 39fb, 39rb | 2 | 728 | 113 | 615 | 0.004 | 0.000 | 1 | 0.371 | 0.227 | 1.703 | 2.039 | 0 |
| Rack1 | 18fb, 18rb | 2 | 560 | 304 | 256 | 0.000 | 0.007 | 3 | 0.087 | 0.052 | 0.627 | 0.578 | 0 |
| **Ran** | 32f, 32r | 1 | 499 | 202 | 297 | 0.011 | 0.003 | 2 | 0.090 | 0.091 | 0.802 | 0.659 | 0 |
| RpL10ab | 19f, 19r | 2 | 955 | 807 | 29 | 0.000 | 0.003 | 3 | 0.072 | 0.043 | 0.641 | 1.001 | 0 |
| RpL13a | 6f, 6r | 2 | 849 | 718 | 131 | 0.000 | 0.019 | 21 | 0.000 | 0.097 | 1.414 | 1.975 | 0 |
| **RpL15** | 2fb, 2rb | 2 | 618 | 412 | 206 | 0.000 | 0.002 | 2 | 0.233 | 0.056 | 1.047 | 1.065 | 16 |
| **RpL27a** | 28fb, 28r | 2 | 501 | 332 | 169 | 0.017 | 0.030 | 16 | 0.155 | 0.101 | 1.309 | 1.078 | 0 |
| **RpL37** | 27f, 27r | 1 | 866 | 785 | 81 | 0.033 | 0.020 | 24 | 0.017 | 0.123 | 1.882 | 2.681 | 0 |
| **RpL37a** | 36f, 36r | 1 | 220 | 91 | 129 | 0.000 | 0.000 | 0 | 0.408 | 0.069 | 1.203 | 0.436 | 0 |
| RpL39 | 16f, 16r | 1 | 463 | 442 | 21 | 0.000 | 0.000 | 0 | 0.000 | 0.086 | 1.386 | 1.055 | 0 |
| **RpS15** | 20fb, 20rb | 1 | 739 | 476 | 263 | 0.058 | 0.035 | 30 | 0.073 | 0.091 | 1.076 | 1.308 | 0 |
| **RpS18** | 22f, 22r | 1 | 812 | 658 | 154 | 0.020 | 0.005 | 6 | 0.072 | 0.052 | 0.757 | 1.011 | 132 |
| **RpS23** | 21f, 21r | 1 | 268 | 79 | 189 | 0.016 | 0.042 | 6 | 0.119 | 0.127 | 0.926 | 0.408 | 0 |
| **RpS4** | 11f, 11r | 1 | 754 | 483 | 271 | 0.000 | 0.000 | 1 | 0.094 | 0.083 | 1.040 | 1.290 | 117 |
| **RpS8** | 5f, 5r | 1 | 422 | 242 | 180 | 0.029 | 0.008 | 6 | 0.060 | 0.034 | 0.447 | 0.311 | 0 |
| **sans_fille** | 35f, 35r | 1 | 446 | 84 | 362 | 0.017 | 0.000 | 2 | 0.140 | 0.037 | 0.501 | 0.367 | 0 |
| **SUI** | 24f, 24r | 1 | 823 | 636 | 186 | 0.000 | 0.006 | 6 | n/a | n/a | n/a | n/a | 0 |
| Tctp | 25f, 25r | 2 | 493 | 148 | 345 | 0.000 | 0.014 | 3 | 0.134 | 0.088 | 0.826 | 0.670 | 0 |
| **Total** | | **28** | **12171** | **7397** | **4774** | | | **136** | | | | | **265** |
| **MEAN per locus** | | | **608.5** | **369.9** | **238.6** | **0.0092** | **0.0105** | **6.8** | **0.1387** | **0.0727** | | | |
| Cox1 | pF2/C2413d | n/a | 698 | n/a | | 0.090 | n/a | 24 | 0.353 | | | | |

## MOLECULAR METHODS

Whole genomic DNA was extracted from specimens stored in 98% ethanol in 50 μl of extraction buffer containing 5% Chelex$^{TM}$100 resin (Bio-Rad, Hercules, CA). To allow for direct sequencing of PCR products without the need to discriminate between haplotypes in heterozygotes, we used males, which are haploid in Hymenoptera, whenever possible. The exceptions were three female *C. fungosa*, for which haplotypes were distinguished by cloning of PCR products as necessary (see below).

Polymerase chain reactions (PCRs) were performed in 20 μl reactions using the following mix for all primer combinations: 2.0 mL 10× Bioline PCR buffer, 2.0 μl bovine serum albumin (10 mg/mL), 0.8 μl MgCl$_2$ (50 mM), 0.16 μl dNTPs (25 mM each), 0.1 μl Taq Polymerase (5 U/μl, Bioline), 0.2 μl of each primer (20 uM), and 1 μl DNA template.

A generic touchdown PCR protocol was used for all loci: 94°C for 3 min, 94°C for 15 sec, an annealing step of 40 sec, 72°C for 3 min, and a final cycle of 72°C for 10 min. The annealing temperature was varied as follows: The first 10 cycles decreased in 1°C increments from 65°C to 55°C, followed by 30 cycles each with an annealing step at 55°C.

To allow comparison of information content in the nuclear loci with a frequently used mitochondrial locus, we also sequenced a 689 bp region of the cytochrome *c* subunit 1 gene (*Cox1*) using primers COI_pF2 and COI_2413d, a modified version of C1-J-2441 (Simon et al. 1994, Table S1). These primers were designed to amplify a fragment largely overlapping the LCO/HCO region of *Cox1* (Folmer et al. 1994), but excluding a poly-T repeat at its 5′ end present in Chalcidoidea, which causes slippage during PCR resulting in uninterpretable sequence.

All PCR products showing single amplified bands were sequenced directly in both directions using ABI BigDye chemistry (Perkin Elmer Biosystems, Waltham, MA) on ABI 3700 and 3730 sequencers in the GenePool Edinburgh. Chromatograms

were checked by eye and complimentary reads aligned using Sequencher version 4.8.

For five loci (*RpS4, RpL27a, RpL37, RpL15b, nAcRbeta*) sequences from female individuals of *C. fungosa* contained putative heterozygous sites or were not readable due to indels. These PCR products were cloned using a mini-Prep kit (Qiagen, Valencia, CA). Five clones were sequenced per locus and individual, one of which was chosen at random for subsequent coalescent analyses. In one case (sample C3, locus *RpS4*) none of the sequenced clones matched the expected product. This sample was excluded from the analysis.

## MODEL OF POPULATION DIVERGENCE AND POPULATION SAMPLING STRATEGIES

We consider a simple model of divergence between three putative refugial populations of *C. fungosa*: Asia Minor and Iran (east, E), Balkans and Central Europe (center, C), and Iberia (west, W). This is analogous to a model of divergence between three species (Takahata et al. 1995; Yang 2002) that has been used to estimate divergence times and ancestral population sizes in Great Apes (Rannala and Yang 2003; Patterson et al. 2006), fruit flies (Villablanca et al. 1998; Li et al. 1999), birds (Jennings and Edwards 2005), and plants (Zhou et al. 2007). The model makes the standard population genetics assumptions of random mating within each population, fixed population sizes between divergence events, and no migration after divergence. The first and last assumptions at least are supported by multilocus allele frequency data for the gallwasp hosts in this system (Stone and Sunnucks 1993; Rokas et al. 2003; Stone et al. 2008).

Following recent studies on Hominids and model organisms (Chen and Li 2001; but see Takahata et al. 1995; Li et al. 1999; Rannala and Yang 2003; Jennings and Edwards 2005; Patterson et al. 2006), we initially adopted a sampling scheme that maximizes the number of loci available by using only a single haploid male from each of the three refugial populations listed above. To examine the impact of increased sampling within populations, we generated an extended dataset, comprising three haploid sequences per population for 13 loci and a single sequence per population for the remaining seven loci as before (Table 1 and Table S2). Impacts of further increases in sample size will be considered based on the theoretical expectation of the number of surviving lineages (Fig. 2).

We used ML (Yang 2002) and Bayesian approaches (Rannala and Yang 2003) (described below) (1) to test whether the most likely order of population divergence is compatible with an "Out of the East" scenario, and (2) to estimate divergence times and ancestral population sizes under this scenario using the single individual per population sampling. To investigate the impact of sample size on parameter estimation, Bayesian analyses were repeated using the extended dataset as defined above (Table S2).

## ALIGNMENT AND MUTATION RATE

*Cecidostiba fungosa* and *C. lauta* sequences were aligned in ClustalW and checked by eye. Exonic regions were assigned by comparison with *D. melanogaster* protein sequences and checked for an open reading frame. Indels in the alignment were treated as missing data.

In the ML and Bayesian analyses, all model parameters are scaled by the per site mutation rate, $\mu$. Conversion of the scaled time between divergence events ($\gamma$) into real times ($\tau$), and of the scaled mutation rate ($\theta$) into effective population sizes ($N_e$), therefore requires an estimate of $\mu$ and its incorporation into the relationships $\gamma = \tau\mu$ and $\theta = 4N_e\mu g$, where $g$ is the average generation time in years. Note that for haplodiploids $N_{e\_hd} = (9N_fN_m)/(2N_f + N_m)$, where $N_f$ and $N_m$ are the number of males and females, respectively, in a randomly mating population. Assuming equal sex ratio and variance in fitness between sexes, $N_{e\_hd}$ is 0.75 $N_{e\_d}$ (Hedrick and Parker 2003).

To calculate a mean estimate of $\mu$ for our loci, we first estimated a synonymous genome-wide mutation rate for the closely related pteromalid wasp genus *Nasonia,* using a divergence time of 0.4 million years ago (mya) between *N. giraulti* and *N. longicornis* (Campbell et al. 1993; Oliveira et al. 2008; Raychoudhury et al. 2009) and a nuclear genome-wide distance at synonymous sites ($K_s$) of 0.011 between these species (Oliveira et al. 2008). With $\mu = K_s/2t$, these values give $\mu = 1.375 \ 10^{-8}$ b/yr. The *Nasonia* divergence time was derived by applying observed bacterial mutation rates to *Wolbachia* symbionts infecting the two *Nasonia* species (Raychoudhury et al. 2009). However, the resulting mutation rate estimate is also remarkably consistent with the few other molecular clock calibrations that exist for insects, such as the calibration of $1.11 \times 10^{-8}$ b/yr for Hawaiian Drosophilids using island ages (Tamura et al. 2004).

To apply the *Nasonia* mutation rate to our intron-rich (and so partially noncoding) sequences, we scaled it by the ratio of the observed average divergence between *C. fungosa* and *C. lauta* at synonymous sites, $K_s$ over the average divergence across all sites $K_{Total}$. This yields a factor of 0.478, so the total average mutation rate for our loci is $\mu = 1.375 \ 10^{-8} \times 0.478 = 6.27 \ 10^{-9}$ b/yr. Note that because this is an average across all sites, it is lower than the mutation rate for synonymous coding sites. This calculation incorporates any mutational constraints on introns and coding sites in *C. fungosa* without making a priori assumptions about intron evolution. We estimated a relative mutation rate for each locus as the observed $K_{Total}$ at each locus over the average $K_{Total}$ (Chen and Li 2001; Yang 2002; Jennings and Edwards 2005), shown in Table 1.

To calculate ancestral effective population sizes, we assumed an average generation time of $g = 0.5$ years for *Nasonia* and *C. fungosa*. This is reasonable for *C. fungosa,* which attacks both sexual spring galls and asexual autumn galls (Askew 1961; Schönrogge et al. 1995, 1996a) (as synonyms *C. adana* and *C. hilaris*), and for temperate populations of *Nasonia*. For comparison with mitochondrial node ages, we calculated a mutation rate for *Cox1* using the JC-corrected distance between *N. giraulti* and *N. longicornis* at this locus and a divergence time of 0.4 mya as before. This gives 22.3% (Oliveira et al. 2008) divergence per site and million years. We compared this locally calibrated clock with estimates obtained in previous studies using the commonly assumed arthropod mitochondrial clock of 2.3% per site and million years (Brower 1994). Despite the obvious shortcomings of the "Brower clock," comparison of relative node ages in this way is valid as long as the same calibration is used across taxa, and a molecular clock assumption is tested and supported in each taxon, as here.

### RECOMBINATION TESTS AND GENE TREE RECONSTRUCTION

Both phylogenetic reconstruction and the coalescent analyses described below make the crucial assumption of no recombination within loci. We determined the minimum number of recombination events using a four-gamete test in DNAsp (Rozas and Rozas 1995) on the largest alignment of each locus. Three loci (*RpS4, RpS18, RpL15*) showed evidence for recombination and were trimmed to the largest nonrecombining block (Galtier et al. 2000; Jennings and Edwards 2005) (shown in Table 1).

Although both the ML and Bayesian approaches described below use site patterns directly and do not rely on estimated gene trees, we reconstructed trees to visualize the data and to test the molecular clock hypothesis that is implicit in both approaches. ML trees were reconstructed for each locus in PAUP* (Swofford 2001). For single individual alignments (triplets), this was done using exact searches, whereas for the three individual per population alignments branch and bound searches were used. Loci varied considerably in relative intron length and hence in base composition. We therefore assumed a single substitution rate but unequal base frequencies (Felsenstein 1981). To test the support for internal nodes in each triplet gene tree, 1000 bootstrap replicates were performed taking a bootstrap value of >70% to indicate strong nodal support (Hillis and Bull 1993). We compared rooting with a strict molecular clock to rooting with *C. lauta* for the triplet gene trees (Tajima 1993; Jennings and Edwards 2005; Tamura et al. 2007). To further test the validity of the molecular clock assumption, we performed Tajima's $1 -$ degree of freedom test on each triplet (Tajima 1993; Jennings and Edwards 2005; Tamura et al. 2007). This nonparametric test is designed for triplet samples given a known species topol-

ogy and is simpler and more powerful than similar model-based tests (Tajima 1993; Nei and Kumar 2000; Jennings and Edwards 2005).

### MAXIMUM LIKELIHOOD ANALYSIS

For minimal sampling, only four parameters in the three-population divergence model matter: the two divergence times $\tau_{C/W}$ and $\tau_{E/C/W}$ and the sizes of the two ancestral populations $N_{C/W}$ and $N_{E/C/W}$ (Fig. 1) and an exact likelihood approach to inference is possible. The program Ne3sML numerically maximizes the likelihood for a given population/species topology (Yang 2002). By default the method assumes an infinite sites mutation model and a molecular clock. Given the level of polymorphism observed in *C. fungosa* (Table 1), this simple model of sequence evolution seems appropriate. For example, if diversity at silent sites (synonymous exon sites and introns) is 0.01 (Table 1), the chance of a back mutation is $10^{-4}$ per site. Because we are analyzing slightly fewer than $10^4$ silent sites in total, we expect to see at most a single back-mutation in the entire dataset and can safely ignore more complicated mutation models.

The likelihood approach of Yang (2002) differs crucially from methods that estimate a species tree conditional on a set of reconstructed gene trees (Degnan and Salter 1995; Maddison and Knowles 2006; Carstens and Knowles 2007; Liu and Pearl 2007; Degnan and Rosenberg 2009; Kubatko et al. 2009) in that it uses the site information directly. The method integrates over all possible gene tree topologies and distributions of branch lengths at each locus and computes the joint log likelihood for a given population history (topology and parameter estimates) as the sum over the log likelihoods of individual loci (Yang 2002; Rannala and Yang 2003). The advantage of this is that in contrast to gene tree species tree approaches (Liu and Pearl 2007; Degnan and Rosenberg 2009; Kubatko et al. 2009) information from unresolved or poorly resolved loci is incorporated automatically. This is particularly important in recently diverged populations. For example, a monomorphic locus resulting from a recent coalescence event would be excluded from analyses conditional on gene tree reconstruction as uninformative, resulting in upwardly biased estimates of divergence time.

We first compared the likelihood of all three possible population tree topologies. Although assessing the statistical significance of nonnested models is difficult in a likelihood setting, models may be ranked by their likelihood (Carstens et al. 2009). Under the "Out of the East" scenario, central and western populations are derived from a shared ancestral population in the center, which in turn split from a common ancestral population in the east, that is, the population tree topology is (E, (C, W)) (Fig. 1). The two alternative topologies are (W, (C, E)), which corresponds to an "Out of the West" scenario, and (C, (E, W)) which is difficult to interpret in the geographic context of *C. fungosa* populations,

because it is unclear where the two ancestral populations would be located.

ML analyses under the most likely population history were performed for two different mutational models. The simplest model assumes a single mutation rate across all loci. We reran this analysis using the relative rates calculated for each locus as described above (Table 1), thereby accounting for possible rate heterogeneity (Table 3).

### BAYESIAN ESTIMATION OF DIVERGENCE TIMES AND ANCESTRAL POPULATION SIZES

MCMCcoal (Rannala and Yang 2003) is the Bayesian equivalent of the ML approach described above. The program uses Markov chain Monte Carlo (MCMC) sampling to estimate posterior probabilities for all model parameters conditional on prior distributions. If multiple individuals per population are sampled, the three population sizes between the present and the most recent divergence event (i.e., $N_E$, $N_C$, $N_W$) (Fig. 1) are modeled as additional parameters. Note that the parameterization in MCMC-coal differs slightly from Ne3sML, as the former uses divergence times rather than internode intervals.

In a Bayesian framework, support for alternative but nonnested models can be compared using Bayes factors (Kass and Raftery 1995). Natural logarithms (ln) of harmonic mean likelihoods (HML) were calculated for each population tree topology (using prior means in analysis a described below) to test support for the "Out of the East" scenario. Following Kass and Raftery (1995), values of twice the difference in lnHML ($2\Delta$lnHML) of 2–6, 6–10, and >10 represent, respectively, positive, strong, and very strong support for the model with higher likelihood.

Because in the case of *C. fungosa* we have no prior knowledge of the model parameters, we used exponentially distributed priors (shape parameter $\alpha = 1$) for all parameters (Jennings and Edwards 2005). To check how sensitive posterior estimates are to prior settings, all analyses were performed twice using different prior means, that is, adjusting $\beta$, the scale parameter of the gamma distribution (Table 4). In the first analysis (*a*) we set prior means to ∼0.150 mya and ∼0.050 mya for $\tau_{E/C/W}$ and $\tau_{C/W}$, respectively ($\beta = 380$) and ∼215,000 for both ancestral population sizes ($\beta = 1520$). In the second analysis (*b*), the prior means for all parameters were increased by an order of magnitude (i.e., changing $\beta$ to 38 and 152) (Table 4). Although the individual parameter values are arbitrary, these two sets of priors should be different enough to assess the robustness of the Bayesian estimation (Jennings and Edwards 2005). Given that incorporating relative mutation rates did not improve estimation using the ML method (see Results), for simplicity all Bayesian analyses were performed assuming a single mutation rate across all loci. Runs were continued for $10^6$ generations with a burn-in of $10^5$

and repeated using different random number seeds to check for convergence.

## Results
### GENE TREES

When only a single individual was sampled from each refugial population, phylogenetic reconstructions for eight of the 18 polymorphic nuclear loci supported the "Out of the East" topology (E, (C, W)) (Fig. 3A), as did the mitochondrial locus *Cox1* (Fig. 2D). Of the remaining loci, two supported each of the two incongruent topologies (Fig. 2B, C) and six showed an unresolved topology (*RpL15, RACK1, ran, Tctp, sansfille, SUI*). Clock-rooted and outgroup-rooted topologies agreed for all resolved loci, but bootstrap support was generally weaker for outgroup rooting (Fig. 3). Although this is not a formal test, the majority of resolved gene trees thus support the "Out of the East" hypothesis (Fig. 1). Tajima's $1 - D$ test rejected a strict molecular clock for only two of 20 loci (*RpS15, RpL 37*). Thus the majority of loci meet the clock assumption implicit in the ML and Bayesian approaches used here.

Increasing our sample size to three individuals from each refugial population resulted in increased variation in gene tree topology (Fig. 4). Despite the many unresolved nodes in some trees, Figure 4 reveals extensive incomplete lineage sorting between *C. fungosa* populations, resulting in a "forest" of largely incongruent gene trees.

### MAXIMUM LIKELIHOOD ANALYSES

The population tree topology (E, (C, W)) had a higher likelihood than either of the two alternative topologies (C, (E, W)) and (W, (C, E)), consistent with the "Out of the East" hypothesis (Table 2). The maximum likelihood estimates (MLEs) of model parameters are broadly consistent between the variable rate (18 loci) and single rate mutational models (using the same 18 loci). However, because the variable rates model has a lower log likelihood, the simpler single rate model was used in all subsequent analyses including the Bayesian runs (Table 3). This also allowed the loci *SUI* and *bellwether*, for which no outgroup sequences could be obtained, to be included in the analyses, giving a total of 20 loci.

Under the "Out of the East" topology (E, (C, W)), the MLE for the older population splitting time between the Iranian population and the ancestor of Hungary and Spain, $\tau_{E/C/W}$, is estimated as 0.110 mya (Table 3). The MLE for $\theta_{E/C/W}$ corresponds to an ancestral population with an effective size of 614,000 before this first split. However, both the MLE for the time between the two population splits, $\tau_{E/C/W} - \tau_{C/W}$ and the population size during that time, $N_{C/W}$ are close to zero, suggesting that Iberian and Hungarian populations may have split almost
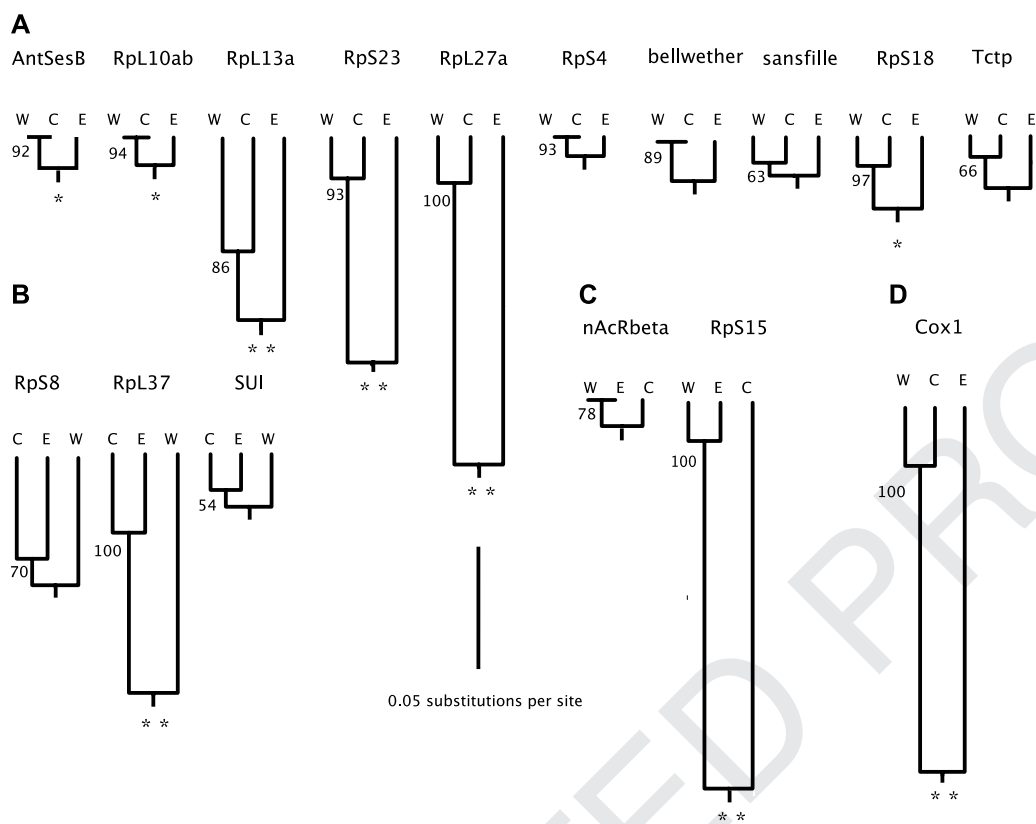
**Figure 3.** ML trees reconstructed for nuclear loci and *Cox1* assuming a strict molecular clock. Bootstrap proportions for the internal node are shown next to each tree. Loci with unresolved topologies (<50% bootstrap support) are not shown. Eight loci have a topology congruent with the "Out of the East" hypothesis (E, (C, W)) (A), two each have topology (W, (C, E)) (B) and (C, (E, W)) (C). The mitochondrial locus *Cox1* is also congruent with "Out of the East" (D). Bootstrap support using rooting with *C. lauta* is indicated with asterisks (* > 50%, ** > 70%) below each tree.

immediately after the initial divergence from the ancestral Eastern population.

### BAYESIAN ESTIMATION OF DIVERGENCE TIMES AND ANCESTRAL POPULATION SIZES

*Minimal sampling*

Bayes factor comparison of lnHML (Table 2) shows that the "Out of the East" model fits the data significantly better than either of the alternative population tree topologies. The contrasting sets of priors *a* and *b* had little impact on posterior estimates of three of the four model parameters (Table 4, Fig. 5A, B, D). Posterior mean ages for the split between eastern populations and the common ancestor of central and western populations $\tau_{E/C/W}$ were 0.118 mya and 0.134 mya in analyses *a* and *b* respectively, with values of 0.043 mya and 0.046 mya for the divide between central and western populations $\tau_{C/W}$ (Table 4). This comparatively long interval between the two divergence times ($\tau_{E/C/W} - \tau_{C/W}$) is in apparent contrast to the results of the ML analysis. However, the 95% confidence intervals for the two divergence times overlap in both prior settings *a* and *b*, such that the lower confidence interval for $\tau_{E/C/W} - \tau_{C/W}$ includes zero, compatible with divergence

between western and central populations occurring immediately after the initial split from the ancestral eastern population. Likewise, the posterior estimate for the effective size of the population ancestral to all three refugial populations ($N_{E/C/W}$) was little influenced by the prior (Table 4, Fig. 5D) (551,000 for *a* and 585,000 for *b*).

In contrast, posterior distributions for the effective size of the population ancestral to central and western populations, $N_{C/W}$, differed considerably between prior settings *a* and *b* (197,000 and 698,000) (Table 4, Fig. 5C). $N_{C/W}$ was also the parameter with the largest variance, the 95% confidence interval spanning two orders of magnitude (priors *b*, Table 4). Notably, with both prior settings, posterior distributions of $N_{C/W}$ peak at the origin (Fig. 5C). This suggests that there is little information about $N_{C/W}$ in the data, with posterior distributions largely reconstructing the prior.

To investigate whether the uncertainty in $N_{C/W}$ can account for the apparent difference in ML and Bayesian estimates of the interval between population splits ($\tau_{E/C/W} - \tau_{C/W}$), we carried out a third MCMCcoal run (Table 4, priors *c*). When the prior mean for $N_{C/W}$ is set to a very low value (2100), the posterior distribution for $\tau_{C/W}$ shifts markedly toward the right (Fig. 5A) such that the
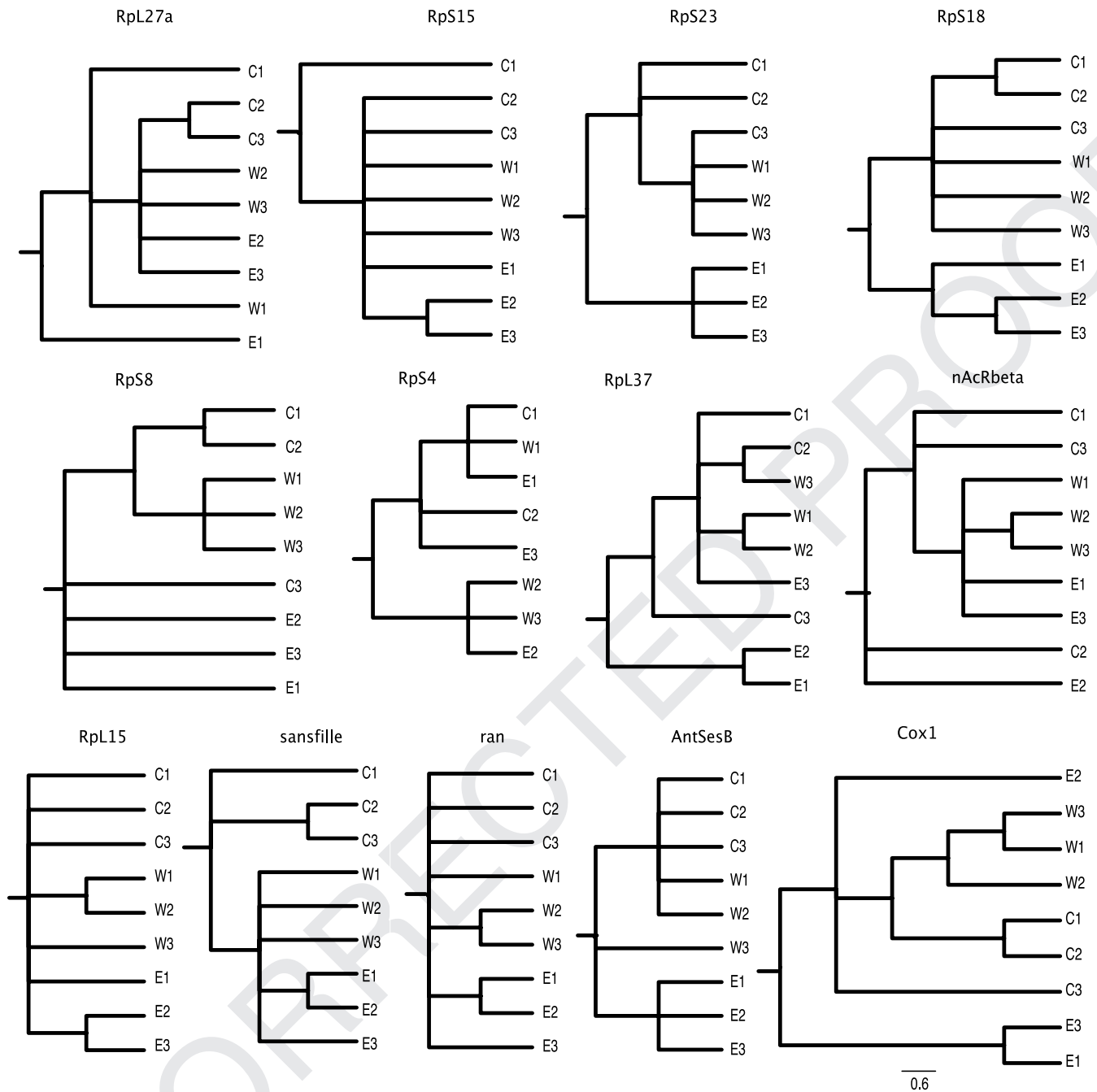
**Figure 4.** ML trees for the extended sampling of three individuals (labeled 1–3) per population for 12 nuclear loci and *Cox1* rooted using *C. lauta*. *RpL37a* is monomorphic and not shown. Although on average, samples from the same population are more closely related than those from different populations, there is extensive lineages sorting, resulting in a "forest" of partially incongruent gene trees.

two divergence events are estimated to have happened in close succession (0.091 and 0.089 mya) in agreement with the ML results (Table 3).

### Extended (three individual) sampling

MCMCcoal analyses of the extended (three individual per population) dataset again gave strongest support to the "Out of the East" scenario (Table 2). Although Bayes factor comparison strongly rejects the "Out of the West" topology (W, (C, E)), the second alternative topology (C, (E, W)) does not provide a significantly worse fit to the data (Table 2).

Parameter estimates agree well with those obtained when only a single individual per population was sampled (Table S3 and Fig. S1). However, increased sampling does have some influence on parameter estimation. First, estimates of $N_{C/W}$, are larger and less sensitive to prior settings when three individuals per

**K. LOHSE ET AL.**

**Table 2.** Comparison of support for alternative population tree topologies, using the lnL of the maximum likelihood estimation (NeML3s) and the harmonic mean likelihood (lnHML) in the Bayesian analyses. In each case the "Out of the East" topology has the highest likelihood (in bold). Values in parentheses show the ln Bayes factor (2ΔlnHML) of the "Out of the East" hypothesis relative to alternatives. Topologies that fit significantly worse than the "Out of the East" hypothesis are indicated with asterisks, using a ln Bayes factor of 2–6 to indicate positive support (*), 6–10 indicate strong support (**), and >10 indicates very strong support (***), following Kass and Raftery (1995).

| | Population tree topology | | |
| --- | --- | --- | --- |
| | Out of the East (E, (C, W)) | Out of the West (W, (C, E)) | (C, (E, W)) |
| NeML3s (single triplet) lnL | **−796.94** | −799.06 | −799.05 |
| MCMCcoal (*a*, single triplet) ln(har.mean) | **−19100.692** | −19103.820 (lnBF=6.25)** | −19103.060 (lnBF=4.73)* |
| MCMCcoal (*a*, extd. triplet) ln(har.mean) | **−19558.237** | −19563.899 (lnBF=11.324)*** | −19558.997 (lnBF=0.76) |

population are sampled for both prior sets *a* and *b* (Table S3). Second, the posterior distributions for $\tau_{C/W}$ are now unimodal, rather than L-shaped with a maximum at the origin (Fig. S1). However, this has little impact on the variance of the posterior. For example, the 95% confidence interval for $\tau_{C/W}$ is 0.005–0.136 mya (priors *a*) in the analysis of the extended samples of three individuals per population, compared with 0.002–0.121 mya in when sampling a single individual (Table 4). Taken together this suggests that increasing sample size per population to three haploid individuals adds some, but not much, power to the estimation of model parameters.

Sampling multiple individuals per population we can also estimate the effective sizes of the three sampled populations between the present and the first divergence events, $N_E$, $N_C$, $N_W$. (Table S3). Although estimates of these parameters had fairly wide confidence intervals and were sensitive to prior settings, their relative magnitude was consistent across analyses. $N_C$ was always the largest followed by $N_E$ and $N_W$. It is also noteworthy that all three estimates were smaller than those obtained for ancestral populations paralleling the findings of Jennings and Edwards (2005) and previous results in Great Ape studies (Chen and Li 2001; Yang 2002; Patterson et al. 2006).

### GENE DIVERGENCE TIMES

Following Jennings and Edwards (2005), we calculated Jukes Cantor distances (D) to estimate coalescence times for each di-

vergence event (D/2) and compared the average distance across loci with the estimated population divergence time and the mitochondrial (*Cox1*) node ages for both single and three individual samples. In both cases, nuclear genes sampled from central and western populations diverged on average almost 0.4 million years (or three glacial periods) prior to the estimated population divergence (Fig. 6). Coalescence times estimated for *Cox1* depend on the assumed mutation rate. Applying the calibration by Oliveira et al. (2008), both coalescence times for *Cox1* (0.013 MY and 0.145 MY respectively) are younger than the average coalescence at nuclear genes but are well within the 95% of the estimated population divergence (Table 4). Using Brower (1994), mitochon- **Q7** drial coalescence between the ancestor of central and western samples and the eastern sample (1.433 mya) predates the average coalescence times for nuclear genes (0.714 mya), whereas the mitochondrial coalescence time between central and western samples (0.125 mya) is still more recent than that for nuclear genes (0.467 mya) (Fig. 6).

## *Discussion*

We analyzed a large multilocus dataset under the simplest possible model of divergence between three populations to make quantitative inferences about the longitudinal history of *C. fungosa*. Reconstructing the genealogical histories of individual loci leads to a "forest" of largely incongruent and often poorly resolved gene

**Table 3.** Maximum Likelihood estimates (MLEs) of ancestral population sizes and population divergence times for refugial populations of *C. fungosa* assuming a population tree topology (E, (C, W)). The simplest mutational model assumes a single rate for all loci. In the variable rates analysis, a relative mutation rate was computed for each locus from divergence to *C. lauta*.

| | MLE, single rate (20 loci) | MLE, single rate (18 loci) | MLE, variable rates (18 loci) |
| --- | --- | --- | --- |
| $\theta_{E/C/W}$ ($N_{E/C/W}$) | 0.0076979 (**614,000**) | 0.007995 (**637,000**) | 0.008933 (**712,000**) |
| $\theta_{C/W}$ ($N_{C/W}$) | 0.000008 (<**1000**) | 0.000002 (<**1000**) | 0.000003 (<**1000**) |
| $\gamma_{E/C/W}$–$\gamma_{C/W}$ (time in My) | 0.0000032 (<**0.001**) | 0.000001 (<**0.001**) | 0.000001 (<**0.001**) |
| $\gamma_{C/W}$ (time in My) | 0.0006924 (**0.110**) | 0.000712 (**0.114**) | 0.000756 (**0.121**) |
| lnL | −853.486 | −794.948 | −796.913 |

**Q6**

**Table 4.** Prior and posterior means and 95% confidence intervals for divergence times and ancestral population sizes in Bayesian analyses using minimal sampling of a single individual per population and assuming an "Out of the East" population tree topology (E, (C, W)). All analyses (*a–c*) assumed exponentially distributed priors (α=1), but differed in their prior means. The population size during the two population divergence times $N_{C/W}$ is the parameter most sensitive to prior choice and has the widest confidence interval.

| Parameter | (α, β) | Prior mean (95% confidence interval) | Posterior mean (95% confidence interval) |
|---|---|---|---|
| **priors *a*** | | | |
| $\theta_{E/C/W}$ | (1, 380) | 0.00271 (0.00011, 0.00968) | 0.00691 (0.00239, 0.01830) |
| $N_{E/C/W}$ | | **216,000 (10,000, 772,000)** | **551,000 (190,000, 1,459,000)** |
| $\theta_{C/W}$ | (1, 380) | 0.00267 (0.00009, 0.00982) | 0.002477 (0.00033, 0.00727) |
| $N_{C/W}$ | | **213,000 (8,000, 783,000)** | **197,000 (26,000, 580,000)** |
| $\gamma_{E/C/W}$ | (1, 1519) | 0.00095 (0.00012, 0.00276) | 0.00074 (0.00019, 0.00139) |
| $\tau_{E/C/W}$ | | **0.151 my (0.019 my, 0.440 my)** | **0.118 my, (0.030 my, 0.221 my)** |
| $\gamma_{C/W}$ | (1, 1519) | 0.000329 (0.00001, 0.00119) | 0.00027 (0.00001, 0.00076) |
| $\tau_{C/W}$ | | **0.052 my, (0.002 my, 0.189 my)** | **0.043 my, (0.002 my, 0.121 my)** |
| **priors *b*** | | | |
| $\theta_{E/C/W}$ | (1, 38) | 0.02664 (0.00083, 0.09691) | 0.00734 (0.00464, 0.01121) |
| $N_{E/C/W}$ | | **2,124,000, (66,000, 7,726,000)** | **585,000 (370,000, 894,000)** |
| $\theta_{C/W}$ | (1, 38) | 0.02639 (0.00064, 0.09669) | 0.00875 (0.00050, 0.05260) |
| $N_{C/W}$ | | **2,104,000 (51,000, 7,709,000)** | **698,000 (40,000, 4,141,000)** |
| $\gamma_{E/C/W}$ | (1, 152) | 0.00980 (0.00113, 0.02918) | 0.00084 (0.00023, 0.00156) |
| $\tau_{E/C/W}$ | | **1.563 my (0.180 my, 4.653 my)** | **0.134 my (0.037 my, 0.249 my)** |
| $\gamma_{C/W}$ | (1, 152) | 0.00326 (0.00008, 0.01198) | 0.00029 (0.00001, 0.00084) |
| $\tau_{C/W}$ | | **0.520 my (0.131 my, 1.910 my)** | **0.046 my (0.002 my, 0.134 my)** |
| **priors *c*** | | | |
| $\theta_{E/C/W}$ | (1, 380) | 0.00257 (0.00004, 0.00961) | 0.00741 (0.00485, 0.01088) |
| $N_{E/C/W}$ | | **205,000 (3,000, 766,000)** | **591,000, (387,000, 868,000)** |
| $\theta_{C/W}$ | (1, 38000) | 0.00003 (<0.00001, 0.00009) | 0.00005 (0.00001, 0.00015) |
| $N_{C/W}$ | | **2,100 (<1000, 7,000)** | **5,000, (<1,000, 13,000)** |
| $\gamma_{E/C/W}$ | (1, 1519) | 0.00096 (0.00011, 0.00277) | 0.00057 (0.00011, 0.00111) |
| $\tau_{E/C/W}$ | | **0.153 my (0.017 my, 0.442 my)** | **0.091 my (0.018 my, 0.177 my)** |
| $\gamma_{C/W}$ | (1, 1519) | 0.00033 (0.00001, 0.00122) | 0.00056 (0.00011, 0.00108) |
| $\tau_{C/W}$ | | **0.053 my (0.013 my, 0.195 my)** | **0.089 my (0.018 my, 0.172 my)** |

trees (Fig. 4), which individually contain little information about the underlying population history. However, analyzing these data jointly in a coalescent framework, the relationship between major refugial populations of *C. fungosa* can be described as a quantified population tree, which includes relevant population genetic parameters (Fig. 7). This is a considerable improvement over previous phylogeographic studies in this system, which have largely been based on mitochondrial sequence data and allozymes (Rokas et al. 2001, 2003; Stone et al. 2001, 2009; Challis et al. 2007) and allows us to quantify important aspects of the phylogeographic history of *C. fungosa*.

First, both likelihood and Bayes factor comparisons of population tree topologies (Table 2) support the "Out of the East" scenario for *C. fungosa*.

Second, both ML and Bayesian estimates for the time of the first population split between the eastern population and the common ancestral population of central and western populations

$\tau_{E/C/W}$ fall well within the late Pleistocene. Likewise, both methods suggest that the more recent divergence between central and western populations ($\tau_{C/W}$) occurred either during the last interglacial or glacial period. However, because the MLE for the time between population splits ($\tau_{E/C/W} - \tau_{C/W}$) is effectively zero and the 95% confidence intervals for the two divergence times overlap in all Bayesian analyses, we cannot exclude the possibility that the two population splits happened in close succession.

Finally, the present coalescent analyses provide information about the effective sizes of ancestral and present populations. Although our estimates of both ancestral population sizes, in particular $N_{C/W}$, have large confidence intervals and, in the case of $N_{C/W}$, are sensitive to prior settings (discussed below), they provide an important comparison with model organisms. For example the observed diversity in *C. fungosa* ($\pi_s = 0.92\%$, Table 1) is comparable with that in non-African populations of *D. melanogaster* ($\pi_s = 1.33\%$) (e.g., Andolfatto 2001, Table 3). Similarly, estimates
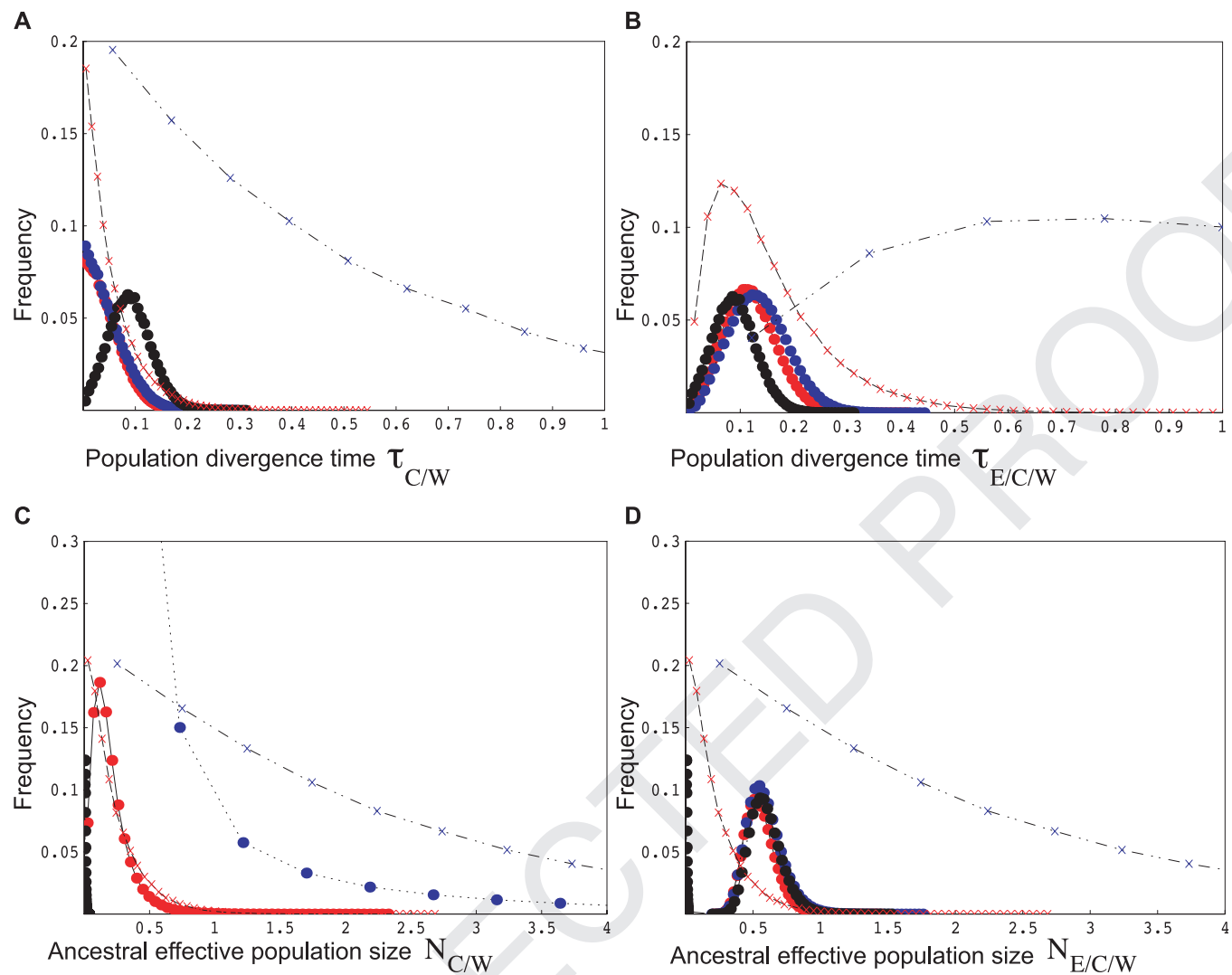
**Figure 5.** Prior and posterior distributions of parameters under the "Out of the East" model of population divergence using minimal sampling of a single individual per population. Prior distributions for the first two MCMCcoal analyses are shown as dashed lines (*a* = narrow, *b* = wide), posterior distributions for the single triplet analysis are in color (*a* = red, *b* = blue and *c* = black). Whereas $\tau_{E/C/W}$ (B) and $N_{E/C/W}$ (D) are little influenced by the prior means, $N_{C/W}$ (C) is extremely sensitive. This parameter is also confounded with $\tau_{C/W}$. When setting a low prior mean for $N_{C/W}$ (analysis *c*) the posterior distribution for $\tau_{C/W}$ shifts markedly toward the right (see black line in A). Note that despite $\alpha = 1$ for all model parameters, the prior distribution for $\tau_{E/C/W}$ (B) is not exponential because of the constraint $\tau_{E/C/W} > \tau_{C/W}$.

for the effective population sizes of *D. melanogaster* of $10^6$ (Andolfatto and Przeworski 2000) and for effective size of the ancestor of *D. melanogaster* and *D. simulans* of $Ng = 3.9 \times 10^5$ (Li et al. 1999) agree with our results for *C. fungosa* in order of magnitude. If effective population sizes of $10^6$ are the rule in insect parasitoids, their longitudinal histories will inevitably involve extensive incomplete lineage sorting, strengthening the case for multilocus approaches for meaningful phylogeographic inferences.

How do these results compare with those obtained from single gene trees both in *C. fungosa* and in other co-distributed oak gall parasitoids and their hosts? In *C. fungosa,* the topology of

the inferred population tree (Fig. 4) is congruent with both the majority of resolved nuclear gene trees as well as the mitochondrial gene tree when a single individual per refugial population was sampled. More generally, the eastern origin of *C. fungosa* is consistent with the mitochondrial gene tree for another oak gall parasitoid, *M. stigmatizans* (Hayward and Stone 2006), with mitochondrial and nuclear gene trees in the parasitoid *M. dorsalis* (Nicholls et al. 2010) and three species of host gall wasps (Rokas et al. 2003; Challis et al. 2007; Stone et al. 2007, 2009).

Although by definition gene divergence must predate the divergence of populations, our results suggest that the magnitude of this difference is considerable in *C. fungosa* and very relevant
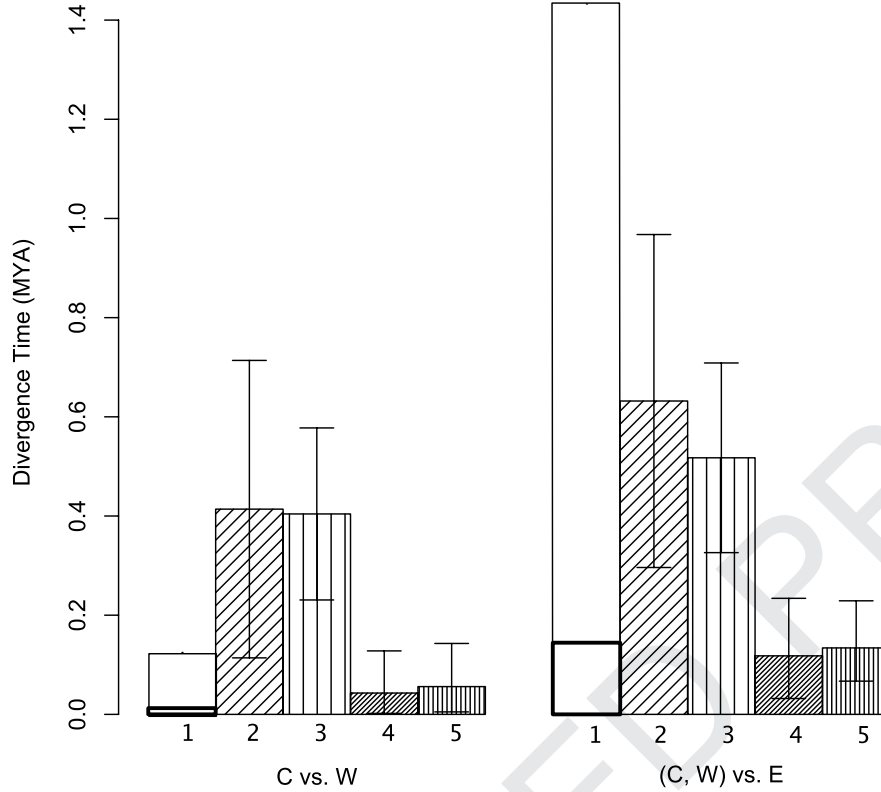
**Figure 6.** Divergence times for the two splits in the Out of the East model (C vs. W left and (C,W) vs. E right). The figure shows that Bayesian estimates (prior settings *a*) of population divergence times for both single and extended triplet samples (columns 4 and 5 in each figure, respectively) are more recent than the mean coalescence time across nuclear loci for both sampling schemes (columns 2 and 3 in each figure). Mitochondrial divergence (column 1) was calculated from node ages in the single triplet tree using both Oliveira et al.'s (2008) rate calibrated from *Nasonia* sister species (lower estimates, bold bars in column 1) and the widely applied rate estimate of Brower (1994) (higher estimates, column 1). Error bars show ±95% confidence limits.

for our interpretation of its Pleistocene history. It is noteworthy that the estimates for $\tau_{E/C/W}$ coincide with the last (Eemian) interglacial 0.130–0.115 mya, which suggests that divergence between refugial populations is as recent as it possibly can be (given the

definition of glacial refugia). We know from the fossil record that both oaks (Velichko et al. 2005) and associated gall wasps species (Stone et al. 2008; van der Ham et al. 2008) known to be attacked by *Cecidostiba* expanded their range in Central and
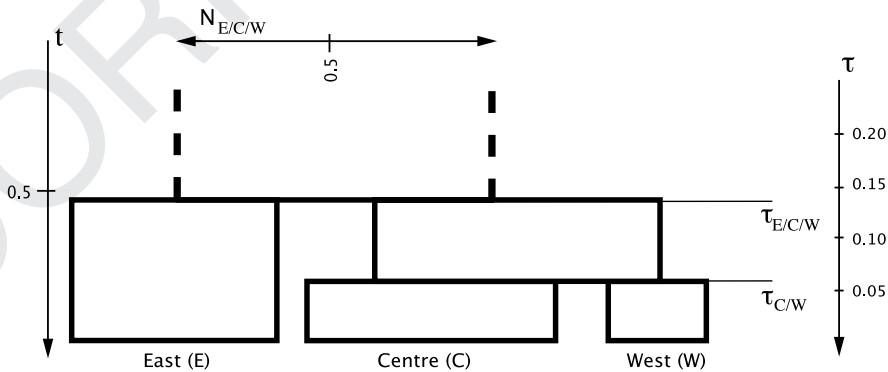


**Figure 7.** Population tree for Western Palearctic *C. fungosa* inferred from 20 genetrees. Means of posterior distributions of model parameters were obtained from the Bayesian analysis (priors *a*, extended sampling of three sequences per population, Table S3 and figure S4). The widths of blocks correspond to effective population sizes (top scale). Divergence times are shown on two different scales: *t* in MY (right-hand scale), and $t = t/(2N_{E/C/W})$ generations assuming two generations per year, that is, $g = 0.5$ (left-hand scale). Note that all blocks have a greater width than height, that is, pairs of lineages sampled from the same population are more likely to coalesce in their ancestral population.

Northern Europe during this period. It is thus plausible for population divergences associated with westwards range expansions of *C. fungosa* to have occurred over a similar timescale.

Although the unknown error in the mitochondrial clock, and the large discrepancy between different calibrations (Brower 1994; Oliveira et al. 2008) make a direct comparison with mitochondrial dates problematic, it is nevertheless reassuring that the mitochondrial ages obtained for *C. fungosa* fall within the 95% confidence interval of (Oliveira et al. 2008) or predate (Brower 1994) the estimated time of population divergence (Fig. 6), as they should. A mitochondrial divergence more recent than that inferred for the population would be inconsistent with the assumed model, and require gene flow between populations. However, it is noteworthy that regardless of the mitochondrial mutation rate used, the *Cox1* divergence times are very different from the average divergence times at nuclear genes (Fig. 6). This demonstrates the extremely large variance in coalescence times and highlights the danger of over-interpreting node ages of single gene trees. An additional problem with mitochondrial mutation rate calibrations is that they are likely to be confounded by the selective dynamics of bacterial endosymbionts (Oliveira et al. 2008), the prevalence of which is known to differ both between populations and closely related species of Pteromalids (Weinert et al. 2009, A. Aebi, unpubl. data). It is therefore not clear to what extent the *Nasonia* rate applies to *C. fungosa*. In contrast, the nuclear estimates for *Nasonia* are broadly consistent with those obtained for other insects.

The fact that divergence at a single locus can only provide an upper bound of the population divergence time may well explain why mitochondrial dates found in previous studies on other species of European gall parasitoids and their gall wasp hosts (Hayward and Stone 2006) are considerably older than the population divergence estimates for *C. fungosa* obtained here. For instance, mitochondrial divergence between Central European and Iberian clades of the parasitoid *M. stigmatizans* has been estimated at 0.264 mya (Hayward and Stone 2006). Mitochondrial divergence estimates between Central Europe and Iberia for gall wasp host species are still older; for example, 0.383 mya in *Andricus kollari* (Hayward and Stone 2006) and 1.6 mya in *Andricus coriarius* sensu stricto (Challis et al. 2007). Analyses of multilocus datasets are clearly required to provide better estimates of population divergence times in these species. As our results show, the fact that the variance in coalescence time is lower for mitochondrial loci given their smaller $N_e$ may reduce but does not alleviate this problem. This underlines the possibility raised by Nichols (2001) that between-taxon variation in mtDNA-inferred dates of divergence between glacial refugia may well be attributable to coalescent variance rather than taxon-specific differences in postglacial dispersal. Rigorous testing of the hypothesis of taxon-specific variation in divergence times requires broader application of multilocus approaches.

## ANCESTRAL $N_e$ AND SAMPLING

The results of the Bayesian analyses show that estimates of $\tau_{C/W}$, or rather the time between the population splits ($\tau_{E/C/W} - \tau_{C/W}$) and the population size during that time, $N_{C/W}$, are confounded. Considering that it is the ratio of the two parameters which determines the chance of coalescence between population splits (Hudson 1983; Saitou and Nei 1986; Yang 2002), this makes intuitive sense and may explain the poor ability to estimate $N_{C/W}$ independently. A large variance in ancestral $N_e$ has also been reported by most earlier multilocus analyses of divergence models (Chen and Li 2001; Yang 2002; Rannala and Yang 2003). In general, explanations for the low power to estimate this parameter fall into two categories: (1) violations of the model assumptions; and (2) limited signal in the data.

Ignoring within-locus recombination and mutational rate heterogeneity, for example, can in principle overestimate ancestral population sizes (Satta et al. 2000; Yang 2002; Wall 2003). However, the few studies that have incorporated these factors suggest that they have little influence on estimates of ancestral $N_e$ (Satta et al. 2000; Yang 2002; Wall 2003). Similarly, the fact that our ML results for the variable mutation model are in agreement with those assuming a single rate despite large differences in relative mutation rates (Table 1) suggests that any impact of mutational heterogeneity between loci is greatly outweighed by coalescence and mutational variance and therefore an unlikely explanation for the low power to estimate $N_{C/W}$.

In general, there are two factors that determine statistical power to infer ancestral parameters; (1) the number of lineages that contribute to the estimate (Fig. 2) and (2) the mutational information available to infer their relationships. Both clearly depend on the timescale of divergence. Relating the estimated population divergence times (scaled by the mean of current population sizes) for *C. fungosa* to the theoretical expectation for the number of surviving lineages, we can ask how much power could potentially be gained by further increasing sample sizes. For example, Figure 2 shows that sampling three instead of a single individual per population roughly doubles the expected number of eastern lineages that survive into the common ancestral population, whereas 16 more individuals are required for a further twofold increase. For the more the recent divergence event at $\tau_{C/W}$, the increase in the number of surviving lineages from additional samples is of course more substantial (Fig. 2). However, if our analysis was limited by sample size, we would expect to see an improvement in parameter estimation proportional to the increase in the number of surviving lineages when sampling three individuals. The fact that this is not the case (i.e., the variance in the estimates of three of the four model parameters is little affected despite the

doubling of surviving lineages) suggests that the power to infer ancestral parameters is largely limited by the mutational variation available rather than the sample size. However, our finding of a markedly higher posterior mean $N_{C/W}$ for the three individual sampling suggests that the estimation of this parameter may indeed be sensitive to the sample size. This makes intuitive sense if we extend the "number of surviving lineage" argument above and consider that only lineages that survive into $N_{C/W}$ and coalesce before they reach $N_{E/C/W}$ contribute to the estimate of $N_{C/W}$. One would therefore expect increased power to estimate this parameter with increasing sample sizes both in *C. fungosa* and in the bird divergence studied by Jennings and Edwards (2005). Thorough investigation of the effect of sampling on statistical power in divergence models both theoretically and using empirical data is required to inform sample designs of future population genetic and phylogeographic studies. In particular, disentangling the effects of mutational limitation and those of sample size (both the number of sampled loci and individuals) would be useful. If mutational information is not limiting, gene tree–species tree methods (Degnan and Salter 1995; Maddison and Knowles 2006; Liu and Pearl 2007; Degnan and Rosenberg 2009; Kubatko et al. 2009) should converge to the same answer as the inference methods used here.

Another way to improve power may be to use outgroup information in the likelihood calculation. At present Ne3sML and MCMCcoal rely on clock rooting (Yang 2002), which, given the small number of polymorphic sites in some loci, results in large topological uncertainty. Being able to distinguish between parsimony informative sites and singleton mutations by reference to an outgroup should in principle enhance the power of both approaches.

## ASSUMPTIONS AND EXTENSIONS OF THE MODEL

Considering the large confidence intervals in parameter estimates, it is clear that quantitative inference of population history is a data-hungry problem, particularly if divergence is recent. It is therefore questionable how much scope there is to probe more realistic models without increasing the amount of data drastically. In general, inferences of ancestral population parameters are likely to be much more sensitive to violations of the divergence model than they are to violations of the model of sequence evolution. Because there are key population processes omitted from the present analyses that render population history less tree-like, one could argue that the notion of a "population tree" as such is an unrealistic description of phylogeographic history.

First, the model assumes that there is no migration after divergence. Although at least in the host gallwasps, allele frequency data support this assumption (Rokas et al. 2001, 2003; Stone et al. 2001, 2008; Challis et al. 2007), we cannot exclude the possibility of migration after divergence for *C. fungosa*. It would therefore be interesting to relax this assumption and IMa, which uses the algorithm of MCMCcoal, has recently been extended to estimate divergence with migration for more than two populations (Hey 2010). However, modeling migration explicitly in a three-population model introduces six additional parameters. Considering the low divergence between *C. fungosa* populations for our loci, there would appear to be little power in the data to distinguish between a divergence model with a very recent split as inferred here and more complicated models involving both divergence and subsequent gene flow. Clearly, much larger amounts of data are needed to successfully explore such models. An additional problem with analyzing models of migration is that, in contrast to strict divergence models, they are sensitive to unsampled populations (Wilkinson-Herbots 2008; Lohse 2009). With the advent of nextgen sequencing technologies, the volumes of data required to explore divergence with gene flow on such recent timescales should soon be available.

Second, the model assumes constant population sizes between divergence events. Again, allowing for changes in population size opens up a myriad of possible historical scenarios and potentially increases the number of parameters dramatically.

Fortunately however, the *C. fungosa* data allow us to at least exclude drastic demographic events. For instance, under a model of colonization through extreme founder events (without subsequent migration), widespread incongruence between gene trees and population trees would not be expected. Thus the mere presence of all possible gene tree topologies in our data allows us to reject this scenario for *C. fungosa*.

And finally, the model assumes panmixia within populations, which may be unrealistic over short timescales and large geographic areas. Recent theoretical work (Slatkin and Pollack 2008) and simulations (Becquet and Przeworski 2009) have demonstrated that subdivision in ancestral populations can lead to misinference under simple divergence models.

In general, any model-based analysis faces the challenge of choosing models that contain sufficient realism to capture key features in the data while being simple enough to be useful. We have shown that in the case of *C. fungosa* a simple divergence model between three populations can explain the observed genetree incongruence and be used to estimate both the origin and divergence time of refugial populations despite the recency of this history. We hope that this study motivates similar analyses of more realistic models.

## TOWARD A MULTILOCUS APPROACH TO COMMUNITY PHYLOGEOGRAPHY

The close ecological dependence of oak gall parasitoids on their hosts and the large number of species involved make this and similar host–parasitoid communities valuable systems in which to study the evolution of ecological interactions (Schönrogge

et al. 1995; Hayward and Stone 2005). Unlike most organisms for which similar multilocus analyses have been conducted (Li et al. 1999; Rannala and Yang 2003; Jennings and Edwards 2005), the ecology of chalcidoid parasitoids involves intricate interactions with co-distributed species at different trophic levels. Linking the extensive information on species composition and food web structure for these communities (Schönrogge et al. 1995, 1996a; Bailey et al. 2009) with population genetic and phylogeographic inferences at the species level opens up an exciting opportunity to address novel and general questions about coevolution and assembly of communities. For instance, do particular lineages or guilds within trophic levels show earlier longitudinal range expansion than others? And if so, what are the ecological properties of such species? For example, are they generalists rather than specialists, and so less likely to go locally extinct (Hayward and Stone 2006)? Further questions arise when considering multiple trophic levels. How correlated are phylogeographic histories between hosts and parasitoids? Is there a general lag between the arrival of gallwasp (or other herbivore) hosts and associated parasitoids such that herbivores experience periods of enemy-free space (Hayward and Stone 2006)? We are currently working on obtaining multilocus data for co-distributed chalcidoid parasitoid species and their gallwasp hosts to address these questions in a quantitative framework. The rarity of many of the species involved (e.g., Schönrogge et al. 1995, 1996a, b, 1998; Stone et al. 1995) means that we will have to make the most of small sample sizes.

Q10

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## *Supporting Information*

The following supporting information is available for this article:

**Figure S1.** Prior and posterior distributions of model parameters under the "Out of the East" scenario of population history obtained for the extended sampling (20 loci, 13 sampled for three individuals per population).

**Table S1.** Primer, sequence, annealing temperature (°C), degeneracy (*De*) for 20 nuclear loci (CG identifier) and *Cox1* used in this study.

**Table S2.** Rearing information and sampling locations of individuals used for sequencing.

**Table S3.** Prior and posterior means and 95% confidence intervals for divergence times and ancestral population sizes in Bayesian analyses of extended sampling (20 loci, 13 sampled for three individuals per population) assuming an "Out of the East" population tree topology (E, (C, W)).

Supporting Information may be found in the online version of this article.

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