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Inferring the direction of introgression using genomic sequence data

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Genomic data are informative about the history of species divergence and interspecific gene flow, including the direction, timing, and strength of gene flow. However, gene flow in opposite directions generates similar patterns in multilocus sequence data, such as reduced sequence divergence between the hybridizing species. As a result, inference of the direction of gene flow is challenging. Here we investigate the information about the direction of gene flow present in multilocus DNA sequence alignments using likelihood-based methods under the multispecies-coalescent-with-introgression (MSci) model. We analyze the case of two species, and use simulation to examine cases with three or four species. We find that it is easier to infer gene flow from a small population to a large one than in the opposite direction, and easier to infer inflow (gene flow from outgroup species to an ingroup species) than outflow (gene flow from an ingroup species to an outgroup species). **It is also easier to infer gene flow if there is a longer time separation between the initial divergence and subsequent introgression.** When introgression is assumed to occur in the wrong direction, the time of introgression tends to be correctly estimated, Bayesian test of gene flow is often significant, while estimates of introgression probability can be even greater than the true probability. We analyze genomic sequences from *Heliconius* butterflies to demonstrate that typical genomic datasets are informative for inferring the direction of interspecific gene flow, as well as its timing and strength.

BPP | direction of gene flow | gene flow | introgression | multispecies coalescent

INTRODUCTION

Gene flow between species is an important evolutionary process that can facilitate species diversification and adaptation (Arnold and Kunte 2017; Campbell et al. 2018; Feurtey and Stukenbrock 2018; Marques et al. 2019; Edelman and Mallet 2021). It occurs as a result of hybridization followed by backcrossing in one of the two hybridizing species. The outcome of introgression in each direction is influenced by multiple factors including mate choice, ecological selection, and hybrid incompatibility (Coyne and Orr 2004; Peters et al. 2017; Martin and Jiggins 2017; Moran et al. 2021). Given that these factors likely differ between species and that selection on introgressed material acts independently in different recipient species it is sensible to assume that gene flow is often asymmetrical, being more prevalent in one direction than in the other. Reliable inference of the direction of introgression, as well as its timing and rate, will advance our understanding of this important evolutionary process and its consequences, including the role of gene flow during speciation and the adaptive

nature of introgressed alleles.

Two models of interspecific gene flow have been developed in the multispecies coalescent (MSC) framework, representing different modes of gene flow (Jiao et al. 2021; Hibbins and Hahn 2022). The MSC-with-introgression (MSci; Flouri et al. 2020) model, also known as multispecies network coalescent (MSNC, Yu et al. 2012; Wen and Nakhleh 2018; Zhang et al. 2018), assumes that gene flow occurs at a particular time point in the past. The magnitude of gene flow is measured by the introgression probability (φ or γ), the proportion of immigrants in the recipient population at the time of introgression. The MSC-with-migration (MSC-M) model, also known as the isolation-with-migration (IM) model, assumes that gene flow occurs continuously at a certain rate every generation after species divergence (Nielsen and Wakeley 2001; Hey et al. 2018). The rate of gene flow is measured by the expected number of immigrants from populations A to B per generation, $M_{AB} = N_B m_{AB}$, where N_B is the (effective) population size of population B and m_{AB} is the proportion of immigrants in population B from A . In both models, the rates

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of gene flow (φ or M) are ‘effective’ rates, reflecting combined effects of gene flow and positive or negative natural selection on introgressed alleles, influenced by the local recombination rate (Westram et al. 2022).

Interspecific gene flow alters gene genealogies, causing fluctuations over the genome in the genealogical history of sequences sampled from extant species. Under both the MSC-M and MSci models, gene trees and coalescent times have probabilistic distributions specified by the model and parameters, including species divergence times, population sizes for extant and extinct species, and the rate of gene flow (see Yang 2014; Jiao et al. 2021 for reviews). Multilocus sequence alignments are informative about gene tree topologies and coalescent times, and thus about the direction of gene flow as well as its timing and strength. However, opposite directions of gene flow often create similar features in gene genealogies and in the sequence data. In the case of two species (say A and B) with one sequence sampled per species per locus, the coalescent time (t_{ab}) between the two sequences (a, b) has the same distribution under the models with $A \rightarrow B$ or $B \rightarrow A$ introgression, so that the direction of introgression is unidentifiable (Yang and Flouri 2022, fig. 10; see also below). However, the direction is identifiable if multiple sequences are sampled per species per locus. When gene flow occurs between non-sister lineages, the direction affects the distributions of the gene trees and coalescent times, and is identifiable even with one sequence per species per locus.

Suppose introgression occurs from species $A \rightarrow B$ but we analyze genomic data assuming $B \rightarrow A$ introgression. (a) Will we often detect introgression despite the assumed wrong direction? (b) How will the estimated introgression probability ($\varphi_{B \rightarrow A}$) compare with the true introgression probability ($\varphi_{A \rightarrow B}$)? (c) How reliable will estimates of the time of introgression be, as well as other parameters such as species divergence times and population sizes? (d) Does the method behavior differ depending on whether gene flow is between sister lineages or between non-sister lineages, and whether gene flow is from a small population to a large one, or in the opposite direction? (e) How can we infer the direction of introgression ($A \rightarrow B$ vs. $B \rightarrow A$)? (f) Are typical genomic data informative about the direction of gene flow? These questions are addressed in this paper. We focus on both Bayesian estimation of parameters (in particular, the introgression probability) (Flouri et al. 2020) and Bayesian test of introgression (Ji et al. 2022). We study the nature of the inference problem and focus on Bayesian inference under the MSci model (Flouri et al. 2020). In comparison, simple summary statistics for inferring gene flow such as D statistic (Green et al. 2010; Durand et al. 2011) may not identify the direction of gene flow. Although heuristic methods exist for inferring the direction of gene flow, based on estimated local genomic divergences (Green et al. 2010: fig. S39)

or genome-wide site-pattern counts (D_{FOIL} , Pease and Hahn 2015), they do not make an efficient use of information in the data, often require a specific species phylogeny and sampling setup, and cannot infer gene flow between sister lineages. See recent discussions of computational strengths and statistical properties of heuristic methods (Jiao et al. 2021; Hibbins and Hahn 2022; Huang et al. 2022; Yang and Flouri 2022).

Here we use a combination of mathematical analysis and computer simulation to characterize features of sequence data that are informative about the direction of gene flow. In the case of two species A and B , we examine the distribution of coalescent times (t_{aa}, t_{ab}, t_{bb}) under the MSci model. The theory allows us to compare and quantify the amount of information in the data under different scenarios. Next, we explore the amount of information gained when a third species is added to branches of the species tree for two species. Finally, we study the impact of introgression direction when gene flow involves non-sister species. Our results provide practical guidelines for inferring introgression and its direction from genomic sequence data. Furthermore, we test these methods with genomic sequences from three species of *Heliconius* butterflies to verify the applicability of results derived from the theoretical analysis and computer simulation and to demonstrate the feasibility of inferring the direction of gene flow, as well as its timing and strength.

RESULTS

Notation and problem setup

We use the MSci model of figure 1a with $A \rightarrow B$ introgression to introduce the notation and set up the problem. Species A and B diverged at time τ_R and hybridized later at time τ_X . The magnitude of introgression is measured by the introgression probability or admixture proportion φ_Y , which is the proportion of immigrants in population B from A at the time of introgression. There are three types of parameters in the model: species divergence times or introgression times (τ_R, τ_X), population sizes for extant and extinct species ($\theta_A, \theta_B, \theta_X, \theta_Y, \theta_R$), and the introgression probability (φ_Y). Divergence time is defined as $\tau = T\mu$, where T is the divergence time in generations and μ is the mutation rate per site per generation. As time T and rate μ are confounded in analysis of sequence data, only τ is estimable. Each branch on the species tree represents a species or population and is associated with a population size parameter, $\theta = 4N_e\mu$, where N_e is the (effective) population size of the species. A branch on the species tree is also referred to by its daughter node so that branch RX is also branch X , with population size θ_X . Both τ and θ are measured in the expected number of mutations per site; i.e., one time unit is the expected time to accumulate one mutation per site. At this time

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scale, coalescence occurs between any two sequences in a population of size θ as a Poisson process with rate $\frac{2}{\theta}$.

Each dataset consists of sequence alignments at L loci, with n_A sequences from A and n_B sequences from B at each locus, and with N sites in each sequence. Underlying the sequences at each locus is a gene tree with branch lengths (coalescent times), with its probability distribution specified by the MSci model (Yu et al. 2014). We make the standard assumptions of no recombination among sites in the sequence of the same locus and free recombination between loci; see Zhu et al. (2022) for a recent simulation examining the impact of recombination on inference under the MSC. Under those assumptions, gene trees and sequence alignments are independent among loci. The data are analyzed under three MSci models that differ in introgression direction: model I with $A \rightarrow B$ introgression, model O with $B \rightarrow A$ introgression, and model B with bidirectional introgression ($A \rightleftharpoons B$) (fig. 1a-c). The ‘inflow’ (I) and ‘outflow’ (O) labels are used here in anticipation of models involving more than two species to be analyzed later. We use the multilocus sequence data to estimate parameters in the MSci model (Flouri et al. 2020). We also use the Bayesian test to detect the presence of gene flow, comparing an MSci model (fig. 1a-c) with the MSC model of no gene flow (fig. 1d) (Ji et al. 2022).

Previously we examined the effects of assigning gene flow onto incorrect branches on the species tree (e.g., parental or daughter branches of the lineage involved in gene flow), and of assuming incorrect modes of gene flow (continuous migration versus episodic introgression) (Huang et al. 2022). Here we examine the effect of misspecified introgression direction.

The case of two species

Distributions of coalescent times and identifiability of introgression direction

We study the distributions of coalescent times between two sequences sampled from the same population (t_{aa}, t_{bb}) or from different populations (t_{ab}). These are analytically tractable and are given in Appendix A. Note that likelihood methods under the MSci model average over the full distribution of the gene tree (G) and coalescent times (t) for sampled sequences at every locus. However, this distribution depends on the sampling configuration (n_A, n_B) and is too complex to analyze. Instead we examine the coalescent times (t_{aa}, t_{ab}, t_{bb}) as important summaries of the data, and use their distributions to demonstrate the identifiability of introgression direction, to characterize the information content in estimation of introgression probability, and to predict the behavior of Bayesian parameter estimation and Bayesian test of gene flow.

First we ask whether introgression direction can

be inferred using sequence data sampled from extant species. From eq. A2, we have $f_i(t_{ab}) = f_o(t_{ab})$ for all $t_{ab} > 0$, with the parameter mapping $\tau_R^{(O)} = \tau_R^{(I)}, \tau_X^{(O)} = \tau_X^{(I)}, \theta_X^{(O)} = \theta_X^{(I)}, \theta_R^{(O)} = \theta_R^{(I)}$ and $\varphi_X = \varphi_Y$, where the superscripts indicate the assumed model (see Yang and Flouri 2022: fig. 10). Thus t_{ab} alone cannot distinguish models I and O. In the case of two species, the direction of introgression is unidentifiable using data of only one sequence per species per locus.

However, introgression direction is identifiable if multiple sequences are sampled from A and B . Information for distinguishing models I and O comes mostly from coalescent times between sequences in the same species (t_{aa}, t_{bb}). If gene flow is $A \rightarrow B$, the coalescent time for sequences from the donor species, t_{aa} , is not affected by the $A \rightarrow B$ introgression. If different populations on the species tree have the same size ($\theta_A = \theta_X = \theta_R$), t_{aa} will have a smooth exponential distribution (e.g., fig. 2a, model I). Otherwise the distribution is discontinuous at time points τ_X and τ_R , because of population size changes. In contrast, t_{bb} has a mixture distribution, depending on the hybridizing species to which each of the two B sequences is traced back on the gene genealogy (i.e., either parental species RX or RY at node Y , fig. 1a). Thus the two models make different predictions about coalescent times t_{aa} and t_{bb} , and the direction of introgression is identifiable when multiple sequences are sampled per species per locus.

If the introgression direction is specified, introgression probability (e.g., φ_Y given model I) is identifiable using data of one sequence per species per locus.

Similarly under model B, even with one sequence per species per locus, introgression probabilities φ_X and φ_Y are identifiable. However, if $\theta_X = \theta_Y$ is assumed in the model, we have $f_B(t_{ab}) = f_I(t_{ab})$ for all t_{ab} if $\varphi_X + \varphi_Y - 2\varphi_X\varphi_Y = \varphi_Y^{(I)}$ (see eq. A4). In other words, under the assumption $\theta_X = \theta_Y$ and with one sequence per species per locus, φ_X and φ_Y in model B are unidentifiable (and only $\varphi_X + \varphi_Y - 2\varphi_X\varphi_Y$ is). Note that model B involves an unidentifiability of the label-switching type, whether one or multiple sequences are sampled from the same species (Yang and Flouri 2022).

Asymptotic analysis and best-fitting parameter values

We consider multilocus datasets generated under model I with $A \rightarrow B$ introgression (fig. 1a) and analyzed under both model I and the misspecified model O with $B \rightarrow A$ introgression. We used four sets of parameter values in model I (fig. 1a) in the numerical calculation, referred to as cases a-d (fig. 2, table S1). When the amount of data (the number of loci) $L \rightarrow \infty$, the maximum likelihood estimates (MLEs) under model I ($\hat{\Theta}_I$) will converge to the true parameter values, with $\hat{\Theta}_I \rightarrow \Theta_I$. Under model O, the MLEs $\hat{\Theta}_O$ will converge to the best-fitting or pseudo-true parameter values (Θ_O^*), which minimize the Kullback-Leibler (KL) divergence from the true

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model to the fitting model: $\hat{\Theta}_O \rightarrow \Theta_O^*$ (e.g., Yang and Zhu 2018). With arbitrary data configurations, it does not seem possible to calculate Θ_O^* analytically. Instead we use as a substitute the averages of posterior means of parameters in BPP analysis of simulated large datasets (with $L = 4000$ loci, $n_A = n_B = 4$ sequences per species per locus and $N = 500$ sites per sequence), shown in table S1. At this data size, average estimates under the true model I are extremely close to the true values, with $\mathbb{E}(\hat{\Theta}_I) \approx \Theta_I$ (table S1), suggesting that the average estimate under model O may also be very close to the infinite-data limits, $\mathbb{E}(\hat{\Theta}_O) \approx \Theta_O^*$. We aim to understand the estimates Θ_O^* by comparing the true distributions of coalescent times under model I, $f_I(t_{aa})$, $f_I(t_{ab})$, and $f_I(t_{bb})$ (eqs. A1–A3), with fitted distributions $f_O(t_{aa})$, $f_O(t_{ab})$, and $f_O(t_{bb})$, calculated using Θ_O^* . **In effect, we treat the true distributions of coalescent times under model I as data, and attempt to derive parameter estimates under the fitting model O to achieve the best fit.**

Our theory is summarized in table 1. Note that parameters τ_R , τ_X , θ_A , θ_B , θ_R in model O are typically well estimated. Introgression time $\tau_X^{(O)}$ is largely determined by the smallest coalescent time between sequences from the two species (t_{ab}), while the discontinuity in the distributions of t_{aa} , t_{ab} , t_{bb} should be informative about $\tau_R^{(O)}$. Thus we expect estimates of those parameters to be close to the true values despite the model misspecification: $\tau_R^{*(O)} \approx \tau_R^{(I)}$ and $\tau_X^{*(O)} \approx \tau_X^{(I)}$. Population sizes $\theta_A^{(O)}$ and $\theta_B^{(O)}$ for the extant species should be well-estimated from multiple samples from the same species, while $\theta_R^{(O)}$ should be well estimated based on coalescent events in the root population. Below we focus on parameters $\theta_X^{(O)}$, $\theta_Y^{(O)}$, and φ_X , which are harder to estimate.

First, by considering the distributions of t_{aa} , we predict $\theta_X^{*(O)} < \theta_X^{(I)}$ (table 1). In the true model I, both A sequences enter X and may coalesce during (τ_X, τ_R) . In the fitting model O, the two A sequences may be separated into different populations due to introgression (one in X and the other in Y), so they may not coalesce in (τ_X, τ_R) as often. Thus having $\theta_X^{*(O)} < \theta_X^{(I)}$ will increase the coalescent rate in X and help to fit model O to $f(t_{aa})$ over (τ_X, τ_R) .

Next from t_{bb} , we predict $\theta_Y^{*(O)} > \theta_Y^{(I)}$ (table 1). In the true model, $A \rightarrow B$ introgression reduces the chance of coalescence between sequences from B during (τ_X, τ_R) . In the fitting model, both B sequences enter Y, leading to a higher chance of coalescence during (τ_X, τ_R) . Thus having $\theta_Y^{*(O)} > \theta_Y^{(I)}$ helps to reduce the chance of coalescence in (τ_X, τ_R) .

Finally, by matching the amount of coalescence between sequences a and b over the time interval (τ_X, τ_R) , or by matching the probability densities $f_I(t_{ab})$ and $f_O(t_{ab})$ for $\tau_X < t_{ab} < \tau_R$, we have approximately

$$\varphi_X^* [1 - e^{-2\Delta\tau/\theta_Y^{*(O)}}] = \varphi_Y [1 - e^{-2\Delta\tau/\theta_X^{(I)}}], \quad (1)$$

where $\Delta\tau = \tau_R - \tau_X$ is assumed to be the same under models I and O based on the arguments above. Eq. 1 predicts that more gene flow will be inferred under model O ($\varphi_X^* > \varphi_Y$) when $\theta_Y^{*(O)} > \theta_X^{(I)}$; if the coalescent rate between sequences a and b during (τ_X, τ_R) is lower in the fitting model than in the true model, a higher φ_X^* than the true φ_Y will increase the chance of such coalescence and achieve a better fit to $f_I(t_{ab})$. Similarly, less gene flow is expected (with $\varphi_X^* < \varphi_Y$) if $\theta_Y^{*(O)} < \theta_X^{(I)}$.

Eq. 1 predicts φ_X^* to be 0.31, 0.35, 0.44 and 0.22 for cases **a–d**, respectively, compared with $\varphi_X^* = 0.27, 0.30, 0.98$ and 0.17 in table S1. The approximation is reasonably good except for case **c**, where φ_X^* was very high. We discuss these cases further in our description of simulation results below.

Simulation results under the true models I and B: parameter estimates have drastically different precisions

To verify and extend our theoretical analysis, we simulated datasets under model I (fig. 1a) and analyzed them under models I, O, and B (fig. 1a–c) using four sets of parameter values. Each dataset consists of $L = 250, 1000$ or 4000 loci, with $n_A = n_B = 4$ sequences sampled per species per locus and $N = 500$ sites in the sequence. Posterior means and 95% highest-probability-density (HPD) credibility intervals (CIs) are plotted in figure 3 (see also table S1 for $L = 4000$).

Model I is the true model, so that the performance under this model constitutes the best-case scenario. Indeed all parameters are well estimated, with the posterior means approaching true values and the CI width approaching 0 when the amount of data $L \rightarrow \infty$ (fig. 3 and table S1, cases **a–d**, model I). Population sizes for extant species (θ_A, θ_B) are much better estimated than those for ancestral species (θ_X, θ_Y). Divergence times (τ_R, τ_X) are well estimated as well. Introgression probability (φ_Y) has substantial uncertainties with wide CIs but with $L = 4000$ loci in the data, the estimates are fairly precise, suggesting that thousands of loci are necessary to estimate introgression probability precisely. These results parallel those found in a previous simulation examining the impact of data size (such as the number of loci, the number sequences per species, and the number of sites) on inference under the MSci model (Huang et al. 2020).

Model B allows bidirectional introgression and thus is a correct model although it is over-parametrized with an extra parameter φ_X . As the amount of data increases, $\hat{\varphi}_Y$ should converge to the true value while $\hat{\varphi}_X$ to 0. Estimates of other parameters are very similar to those under model I, and the CI widths under models I and B are also very similar. In particular, φ_Y is estimated with similar precision in the two models. In large datasets of $L = 4000$ loci, the average CI width is

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0.07, 0.12, 0.08, and 0.16 for cases **a–d** under model I, compared with 0.07, 0.12, 0.09, 0.17 under model B. Even in small or intermediate datasets with $L = 250$ or 1000 loci, the CIs for φ_Y are similar between the two models. Thus over-parametrization incurred little cost to statistical performance of model B. **This may be surprising, because, given the difficulty of inferring introgression direction, one might expect the assumed incorrect $B \rightarrow A$ introgression in model B would interfere with estimation of φ_Y in the correct direction, so that $\hat{\varphi}_Y$ would have a much larger variance under model B than under model I.** However, information concerning φ_Y is largely determined by the number of sequences reaching the hybridization node Y and by the difficulty with which one can tell the parental path taken by each B sequence at Y . Thus there may be little difference in information content about φ_Y between models I and B. Computationally, model B is much more expensive than model I due to sampling an extra parameter in the Markov chain Monte Carlo (MCMC) algorithm and to MCMC mixing issues (Yang and Flouri 2022).

Information content for estimating introgression probability under the true model

Here we consider estimation of introgression probability φ_Y in model I in the four cases (fig. 3, cases **a–d, model I). We characterize the amount of information concerning φ_Y when the correct model is assumed, and explain why φ_Y was much better estimated in case **a** (same θ tall tree) than in case **b** (same θ short tree), and in case **c** (small to large) than in case **d** (large to small) (fig. 3, table S1: cases **a–d**, model I), even though the data size is the same and the true φ_Y is the same (0.2) in all cases. The theory is also useful for understanding later simulation results for larger species trees.**

Consider tracing the genealogical history of sequences at a locus backwards in time. When sequences from B reach the hybridization node Y (fig. 1a), there is a binomial sampling process, with each sequence taking the horizontal (introgression) parental path (into RX) with probability φ_Y and the vertical parental path (into RY) with $1 - \varphi_Y$. However there are two differences from a typical binomial sampling or coin-tossing setup. First, the number of B sequences reaching node Y is a random variable. Second, the outcome of the sampling process (i.e., the parental path taken by the sequence) is not observed but instead reflected in the gene tree and coalescent times (and thus in mutations in the sequences). **Using a coin-tossing analogy, the number of coin tosses is random, and the outcome of the toss is visible only probabilistically.** If a B sequence coalesces with an A sequence during the time interval (τ_X, τ_R) , it will be clear that the B sequence has taken the introgression parental path.

Thus the amount of information in the data concerning φ_Y is determined by **two factors**: (i) the

number of B sequences reaching Y and (ii) the ease with which one can tell the parental path taken by each B sequence at Y . The number of B sequences reaching Y at the locus is given as $n_B - c_B$, where n_B is the number of B sequences sampled at the locus and c_B is the number of coalescent events among them in B before reaching Y . The distribution of $n_B - c_B$ can be easily calculated as a function of n_B and $2\tau_Y/\theta_B$, the length of branch B measured in coalescent units (Tavaré 1984: eqs. 6.1 & 6.2; Wakeley 2009: eqs. 3.39 & 3.41). More B sequences will reach Y the larger n_B is and the smaller $2\tau_Y/\theta_B$ is. For example, it is harder to estimate φ_Y if introgression is older (larger τ_Y).

The second factor — the ease with which one can tell the parental path taken by each B sequence at Y — concerns the probability that two sequences entering X coalesce in X before reaching R ; there is more information about φ_Y the longer the internal branch RX is or the smaller the population size θ_X is (fig. 1a). This may be seen by considering the special case where the data consist of one sequence per species per locus and where the true coalescent time (t_{ab}) is available at each locus. Then the information content for estimating φ_Y may be measured by the Fisher information, given by

$$I_{t_{ab}}(\varphi_Y) \approx \mathbb{E} \left[-\frac{\partial^2}{\partial \varphi_Y^2} \log f_1(t_{ab}) \right] = \frac{P_X}{\varphi_Y(1-\varphi_Y P_X)}, \quad (2)$$

where the expectation is with respect to t_{ab} (eq. A3), and where $P_X = 1 - e^{-\frac{2}{\theta_X}(\tau_R - \tau_X)}$ is the probability that two sequences (a, b) entering population X coalesce in X . Eq. 2 is approximate as it ignores the correlation between parameters and the fact that the Fisher information is in this case a 5×5 matrix (see eq. A3). The asymptotic variance of the estimate ($\hat{\varphi}_Y$) is

$$\mathbb{V}(\hat{\varphi}_Y) \approx \frac{1}{IL} = \frac{\varphi_Y(1-\varphi_Y P_X)}{LP_X} \geq \frac{\varphi_Y(1-\varphi_Y)}{L}, \quad (3)$$

with equality holding if $P_X = 1$. There is thus more information for estimating φ_Y if P_X is greater, or **in other words** if the branch length in coalescent units, $\frac{2}{\theta_X}(\tau_R - \tau_X)$, is greater. Indeed eq. 3 suggests that increasing P_X is more effective in reducing $\mathbb{V}(\hat{\varphi}_Y)$ than increasing the number of loci (L) by the same factor, which is in turn more effective than increasing the number of sampled sequences per locus (n_B) by the same factor.

In our simulation (fig. 3, model I), the introgression probability φ_Y was better estimated in case **a** (same θ tall tree) than in case **b** (same θ short tree). At $L = 4000$, the 95% HPD CI width was 0.07 for case **a**, and 0.12 for case **b**. Consider the two factors. First, in case **a** (tall tree), branch YB is longer, with length $2\tau_Y/\theta_B$ in coalescent units, with a smaller number of sequences reaching Y than in case **b** (short tree). Indeed, given $n_B = 4$ sequences from B , the probability that $n_B - c_B = 1, 2, 3$, and 4 sequences remain by time τ_Y is 0.388, 0.515, 0.095, and 0.002, respectively in case **a**, with an

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average of 1.71 (fig. S1). For the short tree of case **b**, the corresponding probabilities are 0.122, 0.481, 0.347, and 0.050, with average 2.32. The average number of sequences reaching *Y* differ by a factor 1.36. Second, in case **a** (tall tree), any *B* sequence reaching *Y* and taking the left parental path is more likely to coalesce with *A* sequences in *X* than in case **b** (short tree), with $P_X = 1 - e^{-1} = 0.632$ in case **a** and $P_X = 1 - e^{-0.5} = 0.393$ in case **b**, differing by a factor of 1.61. While the two factors have opposite effects, increasing P_X is more effective than increasing $n_B - c_B$, so that φ_Y was far more precisely estimated in case **a** than in **b** (table 3, table S1).

The difference between case **c** (small to large) and case **d** (large to small) was even greater, with φ_Y much better estimated in **c** (fig. 3). At $L = 4000$, the CI width was 0.08 for case **c** and 0.16 for case **d** (table S1). In case **c** more *B* sequences reach *Y* because of the large θ_B than in case **d**. Furthermore *B* sequences reaching *Y* into *X* have a high chance of coalescence with other sequences in population *X*. Both effects make it easier to estimate φ_Y in case **c** than in case **d** (eq. 3). It is thus easier to estimate φ_Y if introgression is from a small population to a large one than in the opposite direction (fig. S2). Note that φ_Y is the proportion of immigrants in the recipient population, so that with the same φ_Y , there are many more migrants in case **c** than in **d**.

Parameter estimation under misspecified introgression direction

When model O was used to analyze data simulated under model I (fig. 1), the introgression direction is misspecified. As discussed above (table 1), species divergence and introgression times (τ_R, τ_X) are well estimated despite misspecification, as are population sizes for extant species and for the root ($\theta_A, \theta_B, \theta_R$). Indeed those parameters are estimated with the same precision under models O and I (fig. 3).

Here we focus on parameters $\varphi_X, \theta_X, \theta_Y$ (fig. 3, model O). Our arguments from the asymptotic analysis (table 1) apply although in simulations the results are affected by random sampling errors due to finite data size.

In cases **a** & **b**, all populations have the same size. Biases in parameter estimates under model O are well predicted by the theory (table 1): based on coalescent times t_{aa}, t_{bb} , and t_{ab} , we expect $\mathbb{E}(\hat{\theta}_X^{(O)}) < \theta_X^{(I)}$, $\mathbb{E}(\hat{\theta}_Y^{(O)}) > \theta_Y^{(I)}$ and $\mathbb{E}(\hat{\varphi}_X) > \varphi_Y$.

In case **c** (small to large), introgression is from a small population to a large one. As the coalescent rate for sequences *a* and *b* over (τ_X, τ_R) is much slower in the fitting model than in the true model, consideration of t_{ab} predicts a large $\hat{\varphi}_X$ or a small $\hat{\theta}_Y^{(O)}$ (table 1). Consideration of t_{bb} suggests $\hat{\theta}_Y^{(O)} > \theta_Y^{(I)}$ will compensate for reduced coalescence between *B* sequences caused by the $A \rightarrow B$ introgression (table

1). Thus predictions about $\hat{\theta}_Y^{(O)}$ based on t_{ab} and t_{bb} are somewhat conflicting. In the simulation, $\hat{\theta}_Y^{(O)}$ is close to $\theta_Y^{(I)}$, much larger than $\theta_X^{(I)}$. The estimate is $\hat{\varphi}_X \approx 100\%$ (table S1). The extreme estimate causes small biases in τ_R and τ_X and poor estimates of $\hat{\theta}_X^{(O)}$ (fig. 3).

Case **d** (large to small) assumes introgression from a large population to a small one (fig. 1a). We expect $\hat{\theta}_X^{(O)} < \theta_X^{(I)}$ based on t_{aa} , and $\hat{\theta}_Y^{(O)} > \theta_Y^{(I)}$ based on t_{bb} (table 1). Moreover, the larger source population in the true model ($\theta_X^{(I)}$) means t_{ab} is less common in (τ_X, τ_R) , with most coalescence occurring in the common ancestor *R*. Thus based on t_{ab} we predict a larger $\hat{\theta}_Y^{(O)}$ or a smaller $\hat{\varphi}_X$ to reduce the amount of coalescence in (τ_X, τ_R) in the fitting model (eq. 1). Thus considerations of both t_{bb} and t_{ab} suggest $\hat{\theta}_Y^{(O)} > \theta_Y^{(I)}$. Depending on whether $\hat{\theta}_Y^{(O)}$ is smaller or greater than $\theta_X^{(I)}$, the introgression probability $\hat{\varphi}_X$ may be greater or smaller than the true φ_Y , according to eq. 1. In our setting, $\hat{\theta}_Y^{(O)} = 0.0107$, slightly greater than $\theta_X^{(I)} = 0.01$, and $\hat{\varphi}_X = 0.17$, slightly smaller than $\varphi_Y = 0.2$ (table S1).

Bayesian test of introgression: power and false positive rate

We applied Bayesian test of gene flow (Ji et al. 2022) to the data analyzed in figure 3. **We are interested in the power of the test under the correct model I. Also we ask how often the test is significant if it is conducted under model O, with introgression direction misspecified.**

Note that the behavior of the test or the asymptotic behavior of posterior probabilities of the compared models is determined by the limiting parameter values when $L \rightarrow \infty$ (Yang and Zhu 2018). If data are simulated under model I (with $\varphi_Y > 0$) and analyzed under model I, the posterior probability for the true model I should approach 1, the Bayes factor in support of model I against model \emptyset (fig. 1d) $B_{I\emptyset} \rightarrow \infty$, and the power of the test should approach 100%, when the data size $L \rightarrow \infty$ (Yang and Zhu 2018). If the data are simulated under model I and analyzed under model B, the power for testing φ_Y (which has the true value $\varphi_Y > 0$) should approach 100%, and the false positive rate for testing φ_X (which has the true value $\varphi_X = 0$) should approach 0, when the data size $L \rightarrow \infty$.

If the data are generated under model I and analyzed under model O, both the null and alternative models are incorrect. According to our analysis $\varphi_X^* > 0$, and model O is a ‘less wrong’ model than model \emptyset , **judged by the Kullback-Leibler divergence** (Yang and Zhu 2018). Thus when $L \rightarrow \infty$, $B_{O\emptyset} \rightarrow \infty$, and the probability of rejecting $H_{\emptyset} : \varphi_X = 0$ will approach 100%. Here the biological interpretation of test results is somewhat ambiguous. If one emphasizes the fact that model O allows gene flow while model \emptyset does not, detecting gene flow may be considered a correct

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result. However, if one emphasizes misspecification of introgression direction in model O, accepting model O may be considered a rather severe false positive error. In this paper, we use the second interpretation.

The MCMC samples generated in BPP runs of figures 3 were processed to calculate the Bayes factor B_{10} in favor of the introgression model (H_1 , fig. 1a-c) against the null MSC model of no gene flow (H_0 , fig. 1d) via the Savage-Dickey density ratio (see Methods). The results are summarized in figure S2, where a 1% significance level was used (i.e., the test is significant if $B_{10} > 100$). When the data were simulated and analyzed under model I and with $L = 250$ loci in the data, power was between 60–100% (fig. S2, cases a-d, model I). In such small datasets, φ_Y was poorly estimated with extremely wide CIs (fig. 3, cases a-d, model I). At $L = 1000$ loci, power was 100% in all four cases. It is thus easier to detect gene flow than to estimate its magnitude reliably. As with our findings on estimation of φ_Y , it is easier to detect gene flow in case a (tall tree) than in case b (short tree), and in case c (small \rightarrow large) than in case d (large \rightarrow small) (fig. S2).

When the data are analyzed under model O, with the introgression direction misspecified, the false positive error is comparable to the power in the analysis under true model I (fig. S2, cases a-d, model O). When the data are analyzed under model B, power to detect the $A \rightarrow B$ introgression is slightly lower than under model I, also reaching 100% at $L = 1000$, while the false positive rate for detecting the non-existent $B \rightarrow A$ introgression is low, below the nominal 1%.

Additional information that results from including a third species

Given two species (A, B) with introgression from $A \rightarrow B$ at the rate of φ (fig. 1a), we consider the information gain for estimating φ from including a third species (C). There are five branches on the two-species tree onto which C can be attached, creating five scenarios (fig. 4a-e): (a) the root population, (b, c) the source and target populations before gene flow, and (d, e) the source and target populations after gene flow. Case c is one of ‘inflow’, with gene flow from the outgroup species (A) into one of the ingroup species (B), while b represents ‘outflow’, with gene flow from an ingroup species (A) into the outgroup (B). Note that in all cases the correct MSci model is used in the analysis, so that the estimate (posterior mean) of φ will converge to the true value (which is 0.2). However, the information content may differ among the five cases. As in the case of two species, the amount of information concerning φ is determined by two factors: (i) the number of sequences reaching the hybridization node and (ii) the ease with which one can tell the parental path taken by each sequence at the hybridization node. When introgression is between nonsister species, information concerning the parental path taken by each sequence may be in the

change of gene-tree topology rather than in the change in between-species coalescent time.

We assumed the same population size $\theta_1 = 0.01$ for all populations, but examined the impact of different population sizes in cases b and c. We simulated 100 replicate datasets in each case. The posterior means, the posterior standard deviation (SD), and the width of the HPD CI for φ are summarized in figure 4f-h. The 95% CIs for other parameters are shown in figure S3.

Equal population sizes on the species tree

If all populations on the species tree have the same size (θ), we expect the amount of information for estimating φ to be in the order a < d < (b, e) < c, with the order of b and e undecided (fig. 4f-h).

First, a < d. Cases a and d are the least informative. Adding an outgroup species C in case a adds little information about φ . In d, the C sequences may reach node X and coalesce with a B sequence in RX, providing information about whether sequences from B take the introgression parental path at node Y. Thus we expect more information in the data in d than in a.

Next, d < b. The number of B sequences reaching node Y is the same in the two cases, so the only difference is in the difficulty of inferring the parental path taken by B sequences at Y. In case b, coalescence of a B sequence with an A sequence causes a change to gene tree topology. In case d, introgression does not cause such topological change to the gene tree. The information content may thus be higher in b than in d.

Next, d < e. In case e, sequences from both B and C may reach the hybridization node Y while in d only sequences from B may reach Y, so that the sample size at node Y is larger (less than twice as large) in e than in d. In d, more sequences enter population RX, increasing slightly the probability of coalescence for any B sequence that takes the introgression parental path at Y, but this effect may be less important than that of increased sample size in e.

Next, b < c (i.e., it is easier to infer inflow than outflow). In both cases, the number of B sequences reaching node Y or the sample size at Y is the same. However, the two cases differ in the ease with which one can tell the parental path taken by each B sequence at Y. In c, coalescence of a B sequence with an A sequence over (τ_X, τ_R) causes a change to gene tree topology. In b such topology change occurs only if the coalescence occurs in the shorter time interval (τ_X, τ_S), and the resulting gene tree is harder to infer because of the shorter internal branch. It is thus harder to resolve the parental path taken by each B sequence at Y in b than in c, and the data are less informative about φ in b. It is harder to infer outflow than inflow.

Finally, e < c. In case c, introgression leads to changes in gene tree topology whereas in e, more sequences reach Y with a larger sample size. The relative effects depend on the parameter values. In the

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simulation here, the increased sample size was less effective than the gene tree topology change (fig. 4g&h, case **c** same- θ vs. case **e**). Note that in **e** the data are more informative about φ the closer τ_S is to τ_Y , and in both **c** and **e** the data are more informative the smaller τ_X is.

Different population sizes on the species tree

For cases **b** (outflow) and **c** (inflow), we also consider different population sizes. The results are shown in figure 4f–h.

First, in case **b**, φ is most poorly estimated in the large→small setting, much better estimated in the same- θ (or large→large) setting, and best in the small→large setting. This can be explained easily by the theory we developed in analysis of the two species case: a large recipient population means many sequences reaching the hybridization node Y and a large sample size, while a small donor species (θ_X) means fast coalescence and easy determination of the parental path taken at node Y . For example, the probability that more than one B sequence reaches Y is 0.613 in case **b** (same θ or small→large), and 0.012 in case **b** (large→small), with a large difference in the sample size.

Similarly in case **c** (inflow), φ is more poorly estimated in the large→small and same- θ (large→large) settings, and was better in the small→large setting. The differences among the three settings are much smaller than in case **b**.

While in the case of same- θ , **b** outflow is less informative than **c** inflow, the order is reversed in the small→large setting (fig. 4). The same number of B sequences reaches node Y in both cases, so the difference must be due to the different levels of difficulty by which one can tell the parental paths taken by B sequences at node Y . In **b**, B sequences taking the introgression parental path go through the small population SX and may coalesce at a high rate with sequences from A (which lead to changes to the gene tree topology informative about introgression), and with sequences from both A and C in population RS . In **c**, B sequences taking the vertical parental path may coalesce in population RS with C sequences, but given that both populations SY and RS are large, this effect may be expected to be minor. While multiple factors can have opposing effects on the relative information content concerning φ in cases **b** versus **c** small→large, on balance, the data are more informative in case **b** than in **c**.

Simulation results in the case of four species

We simulated datasets under the three MSci models of figure 5 for four species on the species tree ((A , (B , C)), D), with introgression between non-sister species A and B in different directions: inflow (I), outflow (O), and bidirectional introgression (B). The

data were analyzed under the same three models (I, O, B), resulting in nine combinations.

Four species, equal population sizes

In case where all populations on the species tree have the same size ($\theta_0 = \theta_1 = 0.01$), average posterior means and 95% HPD CIs are shown in figure 6 and the results for the large datasets of $L = 4000$ are summarized in table S2. Parameters shared among all three models (I, O, B) are well estimated, with no discernible impact of model misspecification. These include species divergence and introgression times (τ_R , τ_S , τ_T , and $\tau_X = \tau_Y$), population sizes for the extant species (θ_A , θ_B , θ_C , θ_D) and for ancestral species not involved in gene flow (θ_R , θ_S). As discussed in the case of two species, introgression time is well estimated even if the introgression direction is misspecified (e.g., τ_X in the I-O and O-I settings), because the estimate is dominated by the minimum sequence divergence between species involved in introgression (t_{ab}) (table 1). Ancestral population sizes (θ_R , θ_S) are slightly less well estimated but appear to converge to the correct values in all settings when the number of loci $L \rightarrow \infty$ (fig. 6). Below we focus on introgression probabilities (φ_X , φ_Y) and population sizes θ_X , θ_Y , and θ_T .

In the I-I, O-O, and B-B settings, the true model is assumed in the analysis, and the results provide a reference for comparison. The introgression probability φ_Y in the I-I setting is more precisely estimated than φ_X in the O-O setting, with narrower CIs. Inflow is easier to infer than outflow, as observed in our simulations for the three-species case (fig. 4b&c, same θ). Similarly in the B-B setting, the inflow probability φ_Y is better estimated than the outflow probability φ_X . The B-B setting had slightly wider CIs for φ_X and φ_Y than in the I-I and O-O settings, due to more parameters in model B (fig. 6, table S2). Overall the introgression probabilities are well estimated under all three settings, although thousands of loci appear necessary to obtain precise estimates. Population size θ_X is better estimated in the I-I setting than in the O-O and B-B settings, because estimation is affected by uncertainties in φ_X in model O and B. Similarly θ_Y is better estimated in O-O than in I-I and B-B settings.

In the I-B and O-B settings, gene flow is unidirectional but the model assumes bidirectional gene flow. The model B is over-parametrized but not misspecified. As Bayesian estimation under the correct model is consistent, the introgression probability for the nonexistent introgression (φ_X in I-B, φ_Y in O-B) should converge to 0 when the data size approaches ∞ . Results in both the I-B and O-B settings are consistent with this expectation (fig. 6). Other parameters are well-estimated, with CI widths indistinguishable from those in the I-I and O-O settings. Over-parametrization in model B incur little cost to the statistical performance of the method, as in the case of two species (fig. 3).

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In the I-O and O-I settings, introgression is assumed to occur in the wrong (opposite) direction. According to our analysis of the two-species case (table 1), this misspecification should only affect estimation of the introgression probability and population sizes θ_X, θ_Y , and θ_T , while other parameters including introgression time should be correctly estimated. This is indeed the case (fig. 6). In the I-O setting, we expect θ_X to be underestimated, θ_Y and θ_T to be overestimated, and the introgression probability φ_X may be larger or smaller than φ_Y depending on how the estimates of θ_Y and θ_T compare with the true value of θ_X (table 1). Simulation results confirm these predictions (fig. 6). In the O-I setting, the effects are the opposite: θ_X was overestimated while θ_Y and θ_T were underestimated. As expected, φ_Y was estimated to be smaller than φ_X .

Finally, in the B-O and B-I settings, introgression occurs in both directions but is assumed to occur in only one direction. The estimates of θ_X, θ_Y , and θ_T follow the same pattern as in I-O and O-I, respectively. The introgression probability (φ_X in B-O, φ_Y in B-I) is larger and less well-estimated than when the true model has unidirectional gene flow (φ_X in O-I, φ_Y in I-O). This positive bias may be explained by the fact that gene flow in the two directions in the true model B have an accumulative effect on the distribution of the coalescent times between species (t_{ab}) (see eq. A4). For instance, in the B-I setting, $B \rightarrow A$ introgression in the true model B is expected to increase the chance of coalescence during $\tau_X < t_{ab} < \tau_S$, and such introgression events may be recognized and misinterpreted as extra $A \rightarrow B$ introgression in the fitting model I, leading to $\hat{\varphi}_Y^{(I)} > \varphi_Y^{(B)}$.

Four species, different population sizes

Results for cases with different population sizes are summarized in figure S4 and table S3. In our setting, model I assumes inflow from a small population to a large one, model O assumes outflow from a large population to a small one, while model B assumes both inflow from small population to large one as well as outflow from large to small (fig. 5).

As in the case of equal population sizes, species divergence and introgression times (τ_R, τ_S, τ_T , and $\tau_X = \tau_Y$) and population sizes for extant species ($\theta_A, \theta_B, \theta_C, \theta_D$) and for common ancestors R and S (θ_R, θ_S) are all well estimated, in spite of model misspecification. Thus we focus on introgression probabilities (φ_X, φ_Y) and population sizes θ_X, θ_Y , and θ_T .

In the I-I, O-O, and B-B settings, the correct model is assumed in the analysis. Introgression probability φ_Y in model I is far more precisely estimated than φ_X in model O. At $L = 4000$ loci, the average 95% CI is 0.19-0.22 for I-I and 0.16-0.24 for O-O (table S3). The difference is far greater than in the case of equal population sizes where the inflow probability φ_Y in model I is

slightly better estimated than the outflow probability φ_X in model O (table S2). It is easier to estimate the introgression probability from a small population to a large one (model I) than in the opposite direction, as discussed before. Similarly in the B-B setting, the inflow probability of small \rightarrow large introgression (φ_Y) is much better estimated than the outflow probability of large \rightarrow small introgression (φ_X): for $L = 4000$, the average 95% CIs are 0.18-0.23 for φ_Y and 0.15-0.24 for φ_X (table S3). In the I-B and O-B settings, model B is over-parameterized. Performance is very similar to that in the I-I and O-O settings, respectively, with φ for the nonexistent migration approaching 0 with the increase of data size (fig. S4, table S3).

In the I-O and O-I settings, the introgression direction is misspecified. In the I-O setting, $\hat{\varphi}_X$ is much greater than in the case of equal population sizes. The extremely large $\hat{\varphi}_X$ mimics the extreme estimate in the two-species case (fig. 3c small \rightarrow large, model O). In the O-I setting, gene flow is from a large population into a small one, and the donor population size θ_Y is grossly underestimated while the recipient population size θ_X is overestimated when introgression direction is misspecified. These patterns are similar to those in the two-species analysis (fig. 3d large \rightarrow small, model O). The estimate $\hat{\varphi}_Y$ is much lower than the true introgression probability $\varphi_X = 0.2$ in the opposite direction.

The B-I and B-O settings show a cumulative effect in the estimates of the migration rate: $\hat{\varphi}_Y$ is greater in the B-I setting than in the I-I setting, and $\hat{\varphi}_X$ is greater in the B-O setting than in the O-O setting. This is the same pattern as found in the case of equal population sizes (fig. 6).

Bayesian test of introgression

We apply the Bayesian test of introgression (Ji et al. 2022) to the data analyzed in figures 6&S4, with results summarized in figures S5&S6. In the I-I, O-O, and B-O settings, where the correct model is assumed, the power of the test is high, reaching $\sim 100\%$ at $L \geq 1000$ loci (figs. S5&S6). In the I-B and O-B settings, the power of detecting introgression in the direction that exists in the true model (B) is high, while the false positive rate for detecting non-existent introgression in the incorrect direction is low, below the nominal 1%. In the I-O and O-I settings, where introgression direction is misspecified, the false positive rate is very high, comparable to the power in the analysis under the correct model. Overall, the results are similar to those for the two-species simulations (fig. S2).

Analysis of *Heliconius* genomic datasets to infer the direction of introgression

To assess the applicability of our results from the asymptotic analysis and simulation to empirical

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datasets and the feasibility of inferring the direction of gene flow using genomic sequence data, we analyzed data from *Heliconius cydno* (*C*), *H. melpomene* (*M*), and *H. hecale* (*H*) (fig. 7). Gene flow is known to occur between *H. cydno* and *H. melpomene*, whereas *H. hecale* is more distantly related, and is here treated as an outgroup, and is assumed not to have had introgression with the other two (Martin et al. 2013). We analyzed coding and noncoding loci on each chromosome as separate datasets (see table S4 for the numbers of loci). We fitted four models: (\emptyset) MSC with no gene flow, (I) MSci with $C \rightarrow M$ introgression, (O) MSci with $M \rightarrow C$ introgression, and (B) MSci with $C \rightleftharpoons M$ bidirectional introgression (see fig. 1). We ran the MCMC algorithm in BPP to generate the posterior estimates of parameters in each model (Flouri et al. 2020) and conducted Bayesian test of introgression (Ji et al. 2022). We describe the results for the coding and noncoding datasets from chromosome 1 (tables 2&3) in detail before discussing results for the other chromosomes.

First, results of the Bayesian test are summarized in table 3. To compare the four different models, we calculated Bayes factors using two approaches: thermodynamic integration with Gaussian quadrature (Lartillot and Philippe 2006; Rannala and Yang 2017) and Savage-Dickey density ratio (Ji et al. 2022); see Materials and Methods. The precise value of the Bayes factor for the same test show some differences depending on the quadrature points in the thermodynamic-integration approach, and is often ∞ by the Savage-Dickey density ratio, reflecting the challenges of calculating the marginal likelihoods or Bayes factors reliably in large datasets (Rannala and Yang 2017). For example $\log B_{I\emptyset}$ for comparison of model I ($C \rightarrow M$ introgression) against model \emptyset (no gene flow) was 1087.1 and 1082.5, respectively, when $K = 32$ and 64 quadrature points were used. This difference is mainly due to the difficulty of calculating the power posterior rather than the use of too few quadrature points (Rannala and Yang 2017). Nevertheless, both values are far greater than the cut-off of 4.6 ($= \log 100$). Similarly the Savage-Dickey density ratio approach estimates $B_{I\emptyset}$ to be ∞ at all three threshold values ($\epsilon = 1\%, 0.1\%, 0.01\%$). Both approaches thus strongly support model I with $C \rightarrow M$ introgression and reject model \emptyset with no gene flow.

For both datasets, the two approaches to Bayes factor calculation lead to the same conclusions, as do the three threshold values for the Savage-Dickey density ratio ($\epsilon = 1\%, 0.1\%, 0.01\%$). The null hypothesis $\varphi_{C \rightarrow M} = 0$ is rejected in the I- \emptyset and B-O comparisons, with strong support for the $C \rightarrow M$ introgression, whether or not the $M \rightarrow C$ introgression is accommodated in the model.

The B-I comparison tests the null hypothesis $\varphi_{M \rightarrow C} = 0$ when both the null and alternative models accommodate the $C \rightarrow M$ introgression. This test leads

to strong support for the null model I, with $B_{BI} < 0.01$. With $C \rightarrow M$ introgression accommodated, the data strongly support the absence of $M \rightarrow C$ introgression. Unlike Frequentist hypothesis testing, which never supports the null strongly, here the Bayesian test strongly favors the null model I, rejecting the more general alternative model B.

However, the test of $\varphi_{M \rightarrow C} = 0$ is significant in the O- \emptyset comparison when the $C \rightarrow M$ introgression is not accommodated in the null and alternative models. This result mimics our computer simulation, in which the test of gene flow is often significant if the assumed gene flow is in the wrong direction (figs. S2, S5&S6).

Models I and O are not nested, but the Bayes factor can be used to compare them. B_{IO} suggests strong preference for model I ($C \rightarrow M$ gene flow) over model O ($M \rightarrow C$ gene flow).

Thus all tests have led to the same conclusions. Both the coding and noncoding datasets strongly support the presence of *H. cydno* \rightarrow *H. melpomene* introgression, and both strongly support the absence of the *H. melpomene* \rightarrow *H. cydno* introgression.

Next, Bayesian parameter estimates under the four models are summarized in table 2. Consistent with the results of the Bayesian test above, estimates of φ under model B (bidirectional introgression) suggest that gene flow is unidirectional. The estimates for the noncoding data are $\hat{\varphi}_{C \rightarrow M} = 0.28$ (95% HPD CI: 0.25–0.31) and $\hat{\varphi}_{M \rightarrow C} < 1\%$ in the opposite direction, while for the coding data, they are $\hat{\varphi}_{C \rightarrow M} = 0.51$ (95% HPD CI: 0.47–0.54) and $\hat{\varphi}_{M \rightarrow C} < 1\%$ (table 2). The reasons for the higher rate ($\hat{\varphi}_{C \rightarrow M}$) for the coding than the noncoding data are unknown. One possible factor may be that introgression was mostly adaptive, driven by natural selection, and that coding loci are under stronger selection. The time of introgression is nearly zero, suggesting that gene flow may be ongoing. Estimates under model I are nearly identical to those under model B. In model O where only $M \rightarrow C$ gene flow is allowed, the introgression probability is estimated to be $\hat{\varphi}_{M \rightarrow C} = 0.17$ (0.15, 0.20) for the noncoding data, and 0.14 (0.08, 0.20) for the coding data. Those rates are substantial, consistent with the significant test results ($B_{O\emptyset}$). Even if gene flow is unidirectional from *C* to *M*, assuming introgression in the opposite (and presumably wrong) direction leads to high estimates of the rate and significant test results. Those results again parallel our simulations (figs. S2, S5 & S6). The misspecified introgression direction in model O causes large estimates of θ_s and reduces τ_s . Those results mimic the behaviors of the misspecified model in the large \rightarrow small case in our theoretical analysis and simulations (fig. 3, table S1d large \rightarrow small).

We note that the divergence time between *H. cydno* and *H. melpomene* (τ_s) is estimated to be much smaller, and θ_s is much larger under model \emptyset (no gene flow) than under models I or B. This is because ignoring gene flow when it occurs leads to underestimation of

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divergence time between species, as expected (Leaché et al. 2014).

Finally, we analyzed the coding and noncoding data from all chromosomes in the same way, with parameter estimates under the four models (Ø, I, O, B) summarized in figure S7 (see also table S5), while Bayesian test results are in table S6.

There is overall consistency among the autosomes (chromosomes 1-20), although estimates of some parameters from chromosomes 5, 10, 13, 15, and 19 appear as outliers. For example, estimates of θ_C and θ_M are unusually large for chromosomes 5, 15, 19, and 20. We discuss the general trends for the autosomes before dealing with chromosome 21.

There is also overall consistency between the coding and noncoding data: divergence and introgression times τ_r and τ_s are nearly proportionally larger for the noncoding than coding data, as was θ_r (S7). This can be explained by a reduced neutral mutation rate for the coding data, due to purifying selection removing nonsynonymous mutations.

Model Ø (no gene flow) underestimated τ_s , the divergence time between the two species involved in gene flow, as expected from previous simulations (Leaché et al. 2014). However, all four models including model Ø produce nearly identical estimates of τ_r , indicating that the impact of introgression is local. Estimates of τ_s under model O are consistently smaller than under models I and B, especially for the coding data, apparently related to the low estimates of $\varphi_{M \rightarrow C}$ for the coding data under model O. Introgression time $\tau_c = \tau_m$ is nearly zero for most chromosomes under all of models I, O, and B, indicating that gene flow may be ongoing (Huang et al. 2022).

Estimates of introgression probability $\varphi_{C \rightarrow M}$ are very similar between models I and B, and they are consistently larger for the coding than noncoding data. Estimates of $\varphi_{M \rightarrow C}$ under model B are consistently ≈ 0 , suggesting the absence of $M \rightarrow C$ gene flow. Estimates of $\varphi_{M \rightarrow C}$ under model O, assuming introgression in the wrong direction, are always larger than estimates under model B, but vary among chromosomes. We suggest that those results are consistent with our simulations (e.g., figs. 3, cases **a–d**), where estimates of introgression probability φ_X in model O vary, even though the true rate in the opposite direction is fixed ($\varphi_Y = 0.2$), influenced by estimates of population sizes such as θ_X and θ_Y .

Results for Z chromosome (chromosome 21) show very different patterns from the autosomes (fig. S7), because we have only one haploid sequence per species in the data (both *H. cydno* and *H. melpomene* samples are hemizygous females, i.e. ZW). For such data, some parameters are unidentifiable in any of the four models, such as $\theta_C, \theta_M, \theta_H$ for the extant species. As discussed before, models I and O are unidentifiable, with $\varphi_M^{(I)} = \varphi_C^{(O)}$ and $\theta_C^{(I)} = \theta_m^{(O)}$. Thus those parameters should have

exactly the same posterior. Under model B, the posterior for φ_M and φ_C are expected to be identical, as are those for θ_c and θ_m . While the age and population size of the root species (τ_r, θ_r) are well estimated, introgression probabilities and ancestral population sizes are poorly estimated with wide CIs. To infer gene flow between species, it is prudent to include multiple samples per species, in particular from the species suspected to be receiving migrants.

Bayes factors calculated via the Savage-Dickey density ratio are presented in table S6. The results are similar to those for chromosome 1, with overwhelming evidence for the $C \rightarrow M$ introgression and no evidence for $M \rightarrow C$ introgression. For some datasets, $B_{O\emptyset} < 100$, so that the test of gene flow ($H_0: \varphi_{M \rightarrow C} = 0$) is not significant when introgression was assumed to be in the wrong direction.

DISCUSSION

Asymmetry of gene flow in nature

No systematic studies have examined the frequency of unidirectional versus bidirectional gene flow given that two species are involved in introgression or hybridization. Both scenarios appear to be common. Sometimes gene flow occurs in one direction even though opportunities exist also in the opposite direction. A well-documented example is gene flow in the *Anopheles gambiae* group of mosquitoes in sub-Saharan Africa (Torre et al. 1997; Slotman, Torre, Calzetta, et al. 2005). Analysis of genomic data provides strong evidence for gene flow from *A. arabiensis* to *A. gambiae* or its sister species *A. coluzzii*, while the rate of gene flow in the opposite direction was estimated to be 0 (Thawornwattana et al. 2018; Flouri et al. 2020). This result from comparisons of genomic sequences is consistent with crossing experiments which supported introgression of autosomal regions from *A. arabiensis* into *A. coluzzii* but not in the opposite direction (Torre et al. 1997; Slotman, Torre, Calzetta, et al. 2005). One possible explanation is that the X chromosome from one species may be incompatible with the autosomal background of the other species (Slotman et al. 2004; Slotman, Torre, and Powell 2005). The *A. arabiensis* \rightarrow *A. gambiae*+*A. coluzzii* introgression is hypothesized to have facilitated the range expansion of *A. gambiae* and *A. coluzzii* into the more arid savanna habitats of *A. arabiensis* (Coluzzi et al. 1979; Ayala and Coluzzi 2005).

Note that the rate of gene flow in the MSci model estimated from the genomic sequence data is an ‘effective’ rate, reflecting the combined effects of gene flow and natural selection. Most introgressed alleles are expected to be purged in the recipient species because of incompatibilities with the host genomic background. It seems likely that alleles at introgressed loci from species *A* on the genomic background of species *B* will

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have different fitnesses than introgressed alleles from *B* on the background of *A*. Another factor is geographic context. If a smaller population of species *A* hybridizes with a larger population of species *B*, *A* is more likely to be swamped by *B*, making introgression asymmetrical. With all those factors considered, one should expect gene flow to be asymmetrical in most systems, with different rates in the two directions.

Gene flow in *Heliconius* butterflies

Heliconius cydno and *H. melpomene* are broadly sympatric across Central America and northwestern South America, and are known to hybridize in the wild (Mallet et al. 2007). Our analysis supports recent unidirectional gene flow from *H. cydno* into *H. melpomene* (fig. 7, tables 2–3, S5, S6), in Panama, where *H. cydno chioneus* and *H. melpomene rosina* are broadly sympatric. In captivity, male F_1 hybrids are fertile while female F_1 hybrids are sterile; male hybrids backcross to either parental species much more readily than the pure species mate with one another (Naisbit et al. 2001; Naisbit et al. 2002).

Previous studies used different approaches to estimate gene flow between these two species. Early phylogenetic analyses of multilocus data attributed recent gene flow between *H. cydno chioneus* and *H. melpomene rosina* as a cause for gene tree variation among loci (Beltrán et al. 2002). An isolation-with-migration (IM) analysis (Hey and Nielsen 2004) using a small number of loci yielded an estimated symmetric bidirectional migration rate m between the two species of 1.7×10^{-6} (95% CI $1.0\text{--}45 \times 10^{-6}$) per generation, with *H. cydno chioneus* having a larger effective population size (Bull et al. 2006). An IM model allowing for different migration rates in each direction found evidence for unidirectional gene flow from *H. cydno* into *H. melpomene*, with $2N_M m_{C \rightarrow M} = 0.294$ (90% HPD CI: 0.116–0.737) whereas $2N_C m_{M \rightarrow C} = 0.000$ (0.000, 0.454) (Kronforst et al. 2006), consistent with our results. Similar patterns were obtained in a subsequent IMa2 analysis (Hey 2010) of a larger dataset (Kronforst et al. 2013). In a more recent analysis of genome-scale data, Martin et al. 2015 estimated a symmetric bidirectional migration rate between *H. c. chioneus* and *H. m. rosina* to be $\hat{M} = 0.20$ (90% HPD interval: 0.09–0.40) per generation. Lohse et al. (2016) compared three models: complete isolation after divergence, and two IM models with unidirectional gene flow, and preferred the model with gene flow from *H. cydno* into *H. melpomene rosina*, with estimated migration rate $4Nm = 1.5$. Martin et al. (2019) used gene tree frequencies to suggest extensive gene flow from *H. cydno* into *H. melpomene* in Panama.

In summary, our results are overall consistent with previous estimates, although our likelihood-based analyses of genomic data provide better estimates and include other important population parameters such as

species divergence times and population sizes (table 2).

Inferring the direction of gene flow using genomic data

If introgression is assumed to occur in the wrong direction, estimates of introgression rate will typically be non-zero, and may even be greater than the true rate. If one tests for introgression and assumes the wrong direction, for example, by using the Bayesian test (Ji et al. 2022), the test may have high power and is often significant. Thus neither a high estimate of introgression rate nor a significant test of introgression is reliable evidence that introgression occurred in the specified direction. Both the high estimates of introgression rate and significant test results for the non-existing introgression may seem surprising and disturbing. Therefore, if there is uncertainty concerning the direction of gene flow, application of the bidirectional model is advisable. Our results suggest that if gene flow is truly unidirectional, over-parameterization of the bidirectional model incurs little cost in statistical performance, as posterior CIs and power to detect gene flow under the bidirectional model are very similar to those under the true unidirectional model. However, this does add to computational cost.

MCMC algorithms can be used to search in the space of introgression models. Currently algorithms that do this (Zhang et al. 2018; Wen and Nakhleh 2018) are not feasible computationally for even small datasets of 100 loci. There is an urgent need for developing efficient MCMC algorithms that are feasible for datasets of thousands of loci. Most approximate methods for detecting gene flow are based on species triplets or quartets and use summaries of sequence data such as genome-wide site-pattern counts (e.g., the *D*-statistic, Green et al. 2010; Durand et al. 2011 and HyDE, Blischak et al. 2018) or gene-tree frequencies (as in SNAQ, Solis-Lemus and Ane 2016). The triplet or quartet trees are then assembled into a large species tree with introgression events (Solis-Lemus et al. 2017). The maximum likelihood method of Yu et al. (2014) uses the estimated gene trees for all species to infer an introgression model. Currently approximate methods do not make an efficient use of information in the multilocus sequence data, and exciting opportunities exist for their improvements (see Degnan 2018; Elworth et al. 2019; Jiao et al. 2021; Hibbins and Hahn 2022 for recent reviews).

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MATERIALS AND METHODS

Asymptotic analysis and simulation in the case of two species

We examined the distributions of coalescent times and conducted computer simulations under model I of figure 1a, with $A \rightarrow B$ introgression. We used four sets of parameter values.

- (a) same θ tall tree: all populations have the same size with $\theta = 0.01$. The other parameters are $\tau_R = \theta$, $\tau_X = 0.5\theta$, and $\varphi_Y = 0.2$.
- (b) same θ short tree: $\theta = 0.01$ for all populations, $\tau_R = 0.5\theta$, $\tau_X = 0.25\theta$, and $\varphi_Y = 0.2$.
- (c) small to large: different species on the species tree have different population sizes, with $\theta_A = \theta_X = \theta_R = \theta_0 = 0.002$ on the left of the tree and $\theta_B = \theta_Y = \theta_1 = 0.01$ on the right, with introgression from a small population to a large one (fig. 1a). Other parameters are $\tau_R = 3\theta_0$, $\tau_X = 1.5\theta_0$ and $\varphi_Y = 0.2$.
- (d) large to small: This is the same as case (c) except that $\theta_A = \theta_X = \theta_R = \theta_0 = 0.01$ on the left of the tree and $\theta_B = \theta_Y = \theta_1 = 0.002$ on the right, so that introgression is from a large population to a small one.

We simulated multilocus sequence datasets under model I (fig. 1a) and analyzed them under models I, O, and B (fig. 1a–c). Each replicate dataset consists of $L = 250, 1000$ or 4000 loci, with $n = 4$ sequences sampled per species per locus. The sequence length is $N = 500$ sites. The `simulate` option of `BPP` (Flouri et al. 2018) was used to simulate gene trees with coalescent times and to ‘evolve’ sequences along the gene tree under the JC model (Jukes and Cantor 1969). Sequences at the tips of the gene tree constitute the data. The number of replicates was 100.

Each replicate dataset was then analyzed using `BPP` (Flouri et al. 2018; Flouri et al. 2020) under models I, O, and B of figure 1a–c. This is the so-called A00 analysis, with the model fixed (Yang 2015). The JC model was assumed in the analysis. Gamma priors were assigned to the age of the root of the species tree (τ_R) and to population size parameters (θ), with the shape parameter $\alpha = 2$ so that the prior was diffuse and with the rate parameter β chosen so that the prior mean was close to the true values. We used $\tau_R \sim G(2, 200)$ and $\theta \sim G(2, 200)$ for case a “same θ tall tree”; $\tau_R \sim G(2, 400)$ and $\theta \sim G(2, 200)$ for case b “same θ short tree”; $\tau_R \sim G(2, 400)$ and $\theta \sim G(2, 400)$ for case c “small to large” and d “large to small”. Introgression probability φ was assigned the beta prior $\text{beta}(1, 1)$, which is $\mathcal{U}(0, 1)$.

MCMC settings were chosen by performing pilot runs, with MCMC convergence assessed by verifying consistency between replicate runs for the same analysis. The same setting was then used to analyze all replicate datasets. We used 16,000 MCMC iterations as burnin, and then took 10^5 samples, sampling every

2 iterations. Running time for analyzing one replicate dataset was ~ 45 mins for $L = 250$ loci or ~ 3 hrs for $L = 1000$ using one thread, and ~ 12 hrs for $L = 4000$ using two threads.

Simulation to evaluate the gain in information for estimating φ by adding a third species

Given the introgression model for two species (A, B) of figure 1a, with $A \rightarrow B$ introgression, we added a third species (C) and assessed the gain in information for estimating φ . There are five branches on the two-species tree, to which the third species could be attached (fig. 4a–e): (a) the root population, (b, c) the source and target populations before gene flow, and (d, e) the source and target populations after gene flow. In all cases $\varphi = 0.2$. The original two-species tree had $\tau_R = \theta_1$ and $\tau_X = \theta_1/2$. In cases b–e, species C was attached to the midpoint of the target branch, while in a, the new root was $1.25\times$ as old as the old root. For models a, d & e, all populations on the species tree had the same size, with $\theta_1 = 0.01$. For cases b and c, three scenarios are considered: (i) equal population size, with $\theta_1 = 0.01$ for all populations; (ii) from small to large, with $\theta_A = \theta_X = \theta_S = \theta_0 = 0.002$ for the thin branches in case b and $\theta_A = \theta_X = \theta_0 = 0.002$ in case c and with $\theta_1 = 0.01$ for all other branches; and (iii) from large to small, with $\theta_B = \theta_Y = \theta_0 = 0.002$ in case b and $\theta_B = \theta_Y = \theta_S = \theta_0 = 0.002$ in case c and with $\theta_1 = 0.01$ for all other branches. For each parameter setting, we simulated 100 replicate datasets. Each dataset consisted of $L = 1000$ loci, with $n_A = n_B = 4$ sequences per species per locus and $N = 500$ sites in the sequence. Each dataset was analyzed using `BPP` to estimate the parameters in the MSci model (fig. 4a–e). Gamma priors were assigned to τ_R and θ : $\tau_R \sim G(2, 200)$ and $\theta \sim G(2, 200)$, while $\varphi_{A \rightarrow B} \sim \mathcal{U}(0, 1)$. We used 32,000 MCMC iterations as burnin, and then took 10^6 samples, sampling every 10 iterations. Running time for analyzing one dataset using one thread was ~ 30 hrs.

Simulation in the case of four species: inflow versus outflow

We simulated data under the three MSci models (I, O, B) of figure 5a–c, with introgression between non-sister species A and B on a four-species tree $((A, (B, C)), D)$. The three models differ in the assumed direction of gene flow, with I for inflow from A to B , O for outflow from B to A , and B for bidirectional introgression between A and B . We used two sets of parameter values. In the first set (same- θ), all species on the tree had the same population size, with $\theta_0 = \theta_1 = 0.01$. In the second set (different- θ), the thin branches had $\theta_0 = 0.002$ while the thick branches had $\theta_1 = 0.01$ (fig. 5a–c). Other parameters were the same in the two settings, with $\tau_R = 4\theta_0$, $\tau_S = 3\theta_0$, $\tau_T = 2\theta_0$, and $\tau_X = \tau_Y = 1.5\theta_0$, and the introgression probabilities were $\varphi_X = \varphi_Y = 0.2$.

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Each dataset consists of $L = 250, 1000$, or 4000 loci, with $n = 4$ sequences per species per locus and with $N = 500$ sites in the sequence. The number of replicates was 100. With three MSci models (I, O, B), two population-size settings (same- θ vs. different- θ), and three data sizes (L), a total of $3 \times 2 \times 3 \times 100 = 1800$ datasets were generated. Each dataset was analyzed under the three models (I, O, B). Gamma priors were assigned to τ_R and θ : $\tau_R \sim G(2, 200)$ and $\theta \sim G(2, 400)$, while $\varphi \sim U(0, 1)$. We used 32,000 MCMC iterations as burnin, and took 2×10^5 samples, sampling every 5 iterations. Running time for analyzing one dataset was ~12hrs for small datasets of $L = 250$ loci and 60hrs for $L = 1000$ using one thread, and ~120hrs for $L = 4000$ using two threads.

Analysis of the *Heliconius* butterfly dataset

We processed the raw genomic sequencing data of Edelman et al. 2019 from three species of *Heliconius* butterflies, *H. hecale* (*H*), *H. cydno* (*C*), and *H. melpomene* (*M*), to retrieve coding and noncoding loci for each chromosome, following the procedure of Thawornwattana et al. (2022). See table S4 for the number of loci in each of the 22 datasets. Each locus consisted of one unphased diploid sequence per species, except the Z chromosome 21 for which only a haploid sequence is available per species (from ZW females). Heterozygote phase in the diploid sequence was resolved using an analytical integration algorithm in the likelihood calculation in BPP (Gronau et al. 2011; Flouri et al. 2018; Huang et al. 2022). We fitted four MSci models with different introgression directions: (\emptyset) MSC with no gene flow, (I) $C \rightarrow M$ introgression, (O) $M \rightarrow C$ introgression, and (B) $C \rightleftharpoons M$ bidirectional introgression.

We assigned priors $\tau_r \sim G(4, 200)$, $\theta \sim G(2, 200)$, and $\varphi \sim U(0, 1)$. We used 10^5 MCMC iterations for burnin, and recorded 10^4 samples, sampling every 100 iterations. For each model, we performed ten independent runs to confirm consistency between runs. The resulting MCMC samples were combined to produce final posterior estimates. Each run took ~100hrs.

Bayesian test of introgression

We applied the Bayesian test of introgression (Ji et al. 2022) to data for two species simulated under the models of figure 1a-c, the data for four species simulated under models I, O, and B of figure 5, and the *Heliconius* datasets (fig. 7).

Bayesian model selection is used to compare the null model of no gene flow $H_0: \varphi = 0$ and the alternative model of introgression $H_1: \varphi > 0$. The Bayes factor $B_{10} = \frac{M_1}{M_0}$, where M_0 and M_1 are marginal likelihood values under H_0 and H_1 . If the prior model probabilities are π_0 and π_1 , B_{10} can be converted into posterior

model probabilities as $\frac{\mathbb{P}(H_1|X)}{\mathbb{P}(H_0|X)} = \frac{\pi_1}{\pi_0} \cdot B_{10}$. If $\pi_0 = \pi_1$, $B_{10} = 100$ will translate to the posterior probability $\mathbb{P}(H_1|X) \approx 1\%$. Thus $B_{10} > 100$ may be considered strong evidence in support of H_1 over H_0 , while $B_{10} < 0.01$ is strong evidence in favor of H_0 over H_1 .

As H_0 and H_1 are nested, B_{10} can be calculated using the Savage-Dickey density ratio (Dickey 1971), by using an MCMC sample under H_1 (Ji et al. 2022). Define an interval of null effects, $\emptyset: \varphi < \epsilon$, inside which the introgression probability is so small that introgression may be considered nonexistent. The Bayes factor in favor of H_1 over H_0 is then

$$B_{10,\epsilon} = \frac{\mathbb{P}(\emptyset)}{\mathbb{P}(\emptyset|X)}, \quad (4)$$

where $\mathbb{P}(\emptyset)$ is the prior probability of the null interval, while $\mathbb{P}(\emptyset|X)$ is the posterior probability, both calculated under H_1 (Ji et al. 2022). Note that $\mathbb{P}(\emptyset) = \mathbb{P}(\varphi < \epsilon) = \epsilon$ if the prior is $\varphi \sim U(0, 1)$. When $\epsilon \rightarrow 0$, $B_{10,\epsilon} \rightarrow B_{10}$ (Ji et al. 2022). We used a few values for ϵ in the range 0.01%–1% to assess its effect. This approach has a computational advantage as it requires running the MCMC under H_1 only and avoids trans-model MCMC algorithms or calculation of marginal likelihood values.

For the *Heliconius* datasets, we in addition used thermodynamic integration combined with Gaussian quadrature to calculate the marginal likelihood under each model, using 32 or 64 quadrature points (Lartillot and Philippe 2006; Rannala and Yang 2017). This approach applies even if the compared models are non-nested, and was used to conduct pairwise comparisons among all four models fitted to the *Heliconius* data.

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APPENDIX A. THE DISTRIBUTION OF COALESCENT TIMES UNDER THE MSCI MODEL FOR TWO SPECIES

Here we gave the probability densities of coalescent times (t_{aa}, t_{ab}, t_{bb}) between two sequences sampled from species A and B under the MSci models I, O, and B of figure 1a–c. These are simple cases of the gene-tree densities given by, e.g., Yu et al. (2014). Example densities under models I and O are plotted in figure 2 for four sets of parameter values.

Under model I,

$$f_I(t_{aa}) = \begin{cases} \frac{2}{\theta_A} e^{-\frac{2}{\theta_A} t_{aa}}, & \text{if } 0 < t_{aa} < \tau_X, \\ e^{-\frac{2}{\theta_A} \tau_X} \frac{2}{\theta_X} e^{-\frac{2}{\theta_X} (t_{aa} - \tau_X)}, & \text{if } \tau_X < t_{aa} < \tau_R, \\ e^{-\frac{2}{\theta_A} \tau_X} e^{-\frac{2}{\theta_X} (\tau_R - \tau_X)} \\ \quad \times \frac{2}{\theta_R} e^{-\frac{2}{\theta_R} (t_{aa} - \tau_R)}, & \text{if } t_{aa} > \tau_R. \end{cases} \quad (A1)$$

This is a function of $\tau_R, \tau_X, \theta_A, \theta_X, \theta_R$, independent of $\theta_B, \theta_Y, \varphi_Y$. From the viewpoint of the two A sequences, there are demographic changes in population size with $\theta_A, \theta_X, \theta_R$, respectively, for the three time segments $(0, \tau_X)$, (τ_X, τ_R) , and (τ_R, ∞) .

The coalescent time between two sequences sampled from species B has the distribution

$$f_I(t_{bb}) = \begin{cases} \frac{2}{\theta_B} e^{-\frac{2}{\theta_B} t_{bb}}, & \text{if } 0 < t_{bb} < \tau_X, \\ e^{-\frac{2}{\theta_B} \tau_X} \left[(1 - \varphi_Y)^2 \frac{2}{\theta_Y} e^{-\frac{2}{\theta_Y} (t_{bb} - \tau_X)} \right. \\ \quad \left. + \varphi_Y^2 \frac{2}{\theta_X} e^{-\frac{2}{\theta_X} (t_{bb} - \tau_X)} \right], & \text{if } \tau_X < t_{bb} < \tau_R, \\ e^{-\frac{2}{\theta_B} \tau_X} \left[(1 - \varphi_Y)^2 e^{-\frac{2}{\theta_Y} (\tau_R - \tau_X)} \right. \\ \quad \left. + \varphi_Y^2 e^{-\frac{2}{\theta_X} (\tau_R - \tau_X)} + 2\varphi_Y(1 - \varphi_Y) \right] \\ \quad \times \frac{2}{\theta_R} e^{-\frac{2}{\theta_R} (t_{bb} - \tau_R)}, & \text{if } t_{bb} > \tau_R. \end{cases} \quad (A2)$$

This is a function of $\tau_R, \tau_X, \theta_B, \theta_X, \theta_Y, \theta_R, \varphi_Y$, and is independent of θ_A . In the time interval $(0, \tau_X)$, coalescence between the two B sequences occurs at the rate $2/\theta_B$, as when there is no gene flow. There is a suppression of coalescent events in the interval (τ_X, τ_R) , due to introgression, as no coalescence is possible if one of the two B sequences migrates into X (with time running backwards).

Finally

$$f_I(t_{ab}) = \begin{cases} \varphi_Y \frac{2}{\theta_X} e^{-\frac{2}{\theta_X} (t_{ab} - \tau_X)}, & \text{if } \tau_X < t_{ab} < \tau_R, \\ \left[(1 - \varphi_Y) + \varphi_Y e^{-\frac{2}{\theta_X} (\tau_R - \tau_X)} \right] \\ \quad \times \frac{2}{\theta_R} e^{-\frac{2}{\theta_R} (t_{ab} - \tau_R)}, & \text{if } t_{ab} > \tau_R. \end{cases} \quad (A3)$$

This is a function of $\tau_R, \tau_X, \theta_X, \theta_R$, and φ_Y , and is independent of $\theta_A, \theta_B, \theta_Y$. Coalescence between a and b may occur during (τ_X, τ_R) if the B sequence migrates into A (with time running backwards).

Under model O with $B \rightarrow A$ introgression (fig. 1b), $f_O(t_{aa}), f_O(t_{ab})$, and $f_O(t_{bb})$ are given by $f_I(t_{bb})$,

$f_I(t_{ab})$, and $f_I(t_{aa})$ with a change of symbols.

Under model B with both $A \rightarrow B$ and $B \rightarrow A$ introgressions (fig. 1c), $f_B(t_{aa}) = f_O(t_{aa})$ and $f_B(t_{bb}) = f_I(t_{bb})$, while

$$f_B(t_{ab}) = \begin{cases} (1 - \varphi_X) \varphi_Y \frac{2}{\theta_X} e^{-\frac{2}{\theta_X} (t_{ab} - \tau_X)} \\ \quad + \varphi_X (1 - \varphi_Y) \frac{2}{\theta_Y} e^{-\frac{2}{\theta_Y} (t_{ab} - \tau_X)}, & \text{if } \tau_X < t_{ab} < \tau_R, \\ \left[(1 - \varphi_X)(1 - \varphi_Y) + \varphi_X \varphi_Y \right. \\ \quad \left. + (1 - \varphi_X) \varphi_Y e^{-\frac{2}{\theta_X} (\tau_R - \tau_X)} \right. \\ \quad \left. + \varphi_X (1 - \varphi_Y) e^{-\frac{2}{\theta_Y} (\tau_R - \tau_X)} \right] \\ \quad \times \frac{2}{\theta_R} e^{-\frac{2}{\theta_R} (t_{ab} - \tau_R)}, & \text{if } t_{ab} > \tau_R. \end{cases} \quad (A4)$$

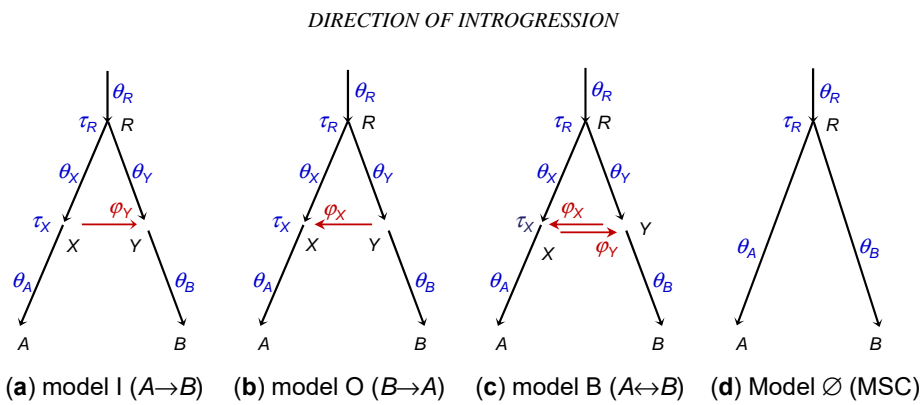


Figure 1: (a–c) MSci models for two species with different directions of gene flow showing model parameters: (a) $A \rightarrow B$ introgression (I for ‘inflow’) with $\Theta_I = (\tau_R, \tau_X, \theta_A, \theta_B, \theta_X, \theta_Y, \theta_R, \varphi_Y)$, (b) $B \rightarrow A$ introgression (O for ‘outflow’) with $\Theta_O = (\tau_R, \tau_X, \theta_A, \theta_B, \theta_X, \theta_Y, \theta_R, \varphi_X)$, or (c) bidirectional introgression (B) with $\Theta_B = (\tau_R, \tau_X, \theta_A, \theta_B, \theta_X, \theta_Y, \theta_R, \varphi_X, \varphi_Y)$. The magnitude of introgression is measured by the introgression probability: $\varphi_Y \equiv \varphi_{A \rightarrow B}$ in a and c or $\varphi_X \equiv \varphi_{B \rightarrow A}$ in b and c. Note that horizontal arrows (XY and YX) represent introgression events rather than real populations and have no θ associated with them. (d) MSC model with no gene flow, with $\Theta_\emptyset = (\tau_R, \theta_A, \theta_B, \theta_R)$.

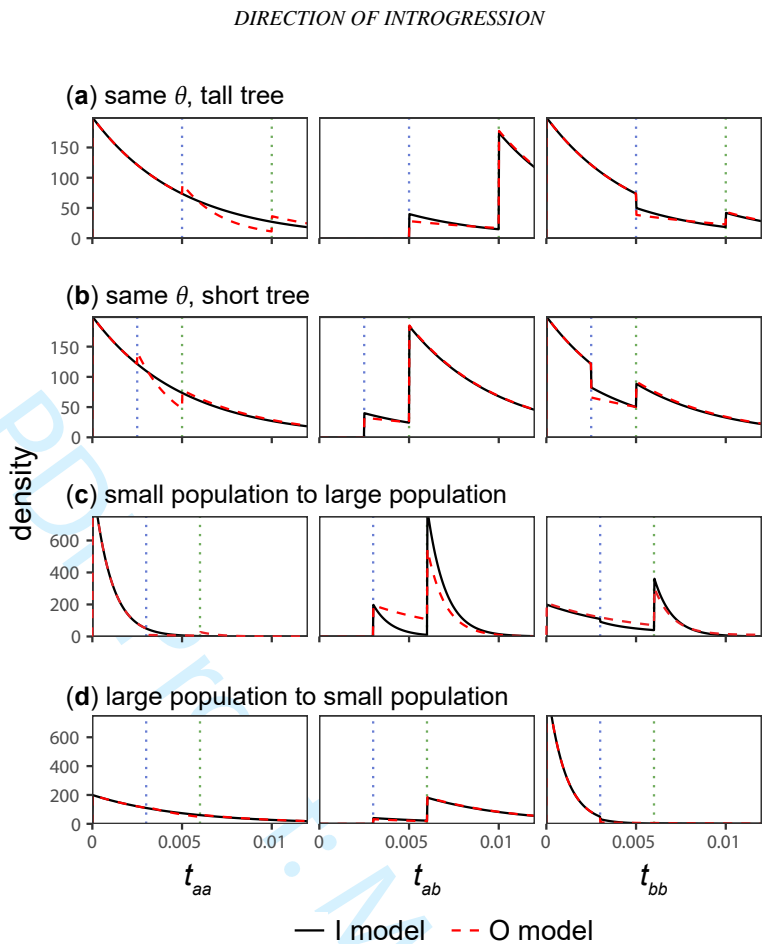


Figure 2: The true (black solid line for model I) and fitted (red dashed line for model O) distributions of coalescent times (t_{aa}, t_{ab}, t_{bb}) for four sets of parameter values (cases **a–d**). Data are generated under model I and analyzed under model O of figure 1**a–b**. Densities for model I are calculated using the true parameter values (Θ_I in table S1); see eqs. A1–A3, while those for model O are calculated using the best-fitting parameter values, approximated by average estimates in BPP analysis of simulated large datasets (of $L = 4000$ loci, $n = 4$ sequences per species per locus and $N = 500$ sites in the sequence) (Θ_O^* in table S1). Vertical dotted lines indicate discontinuity points at τ_X and τ_R .

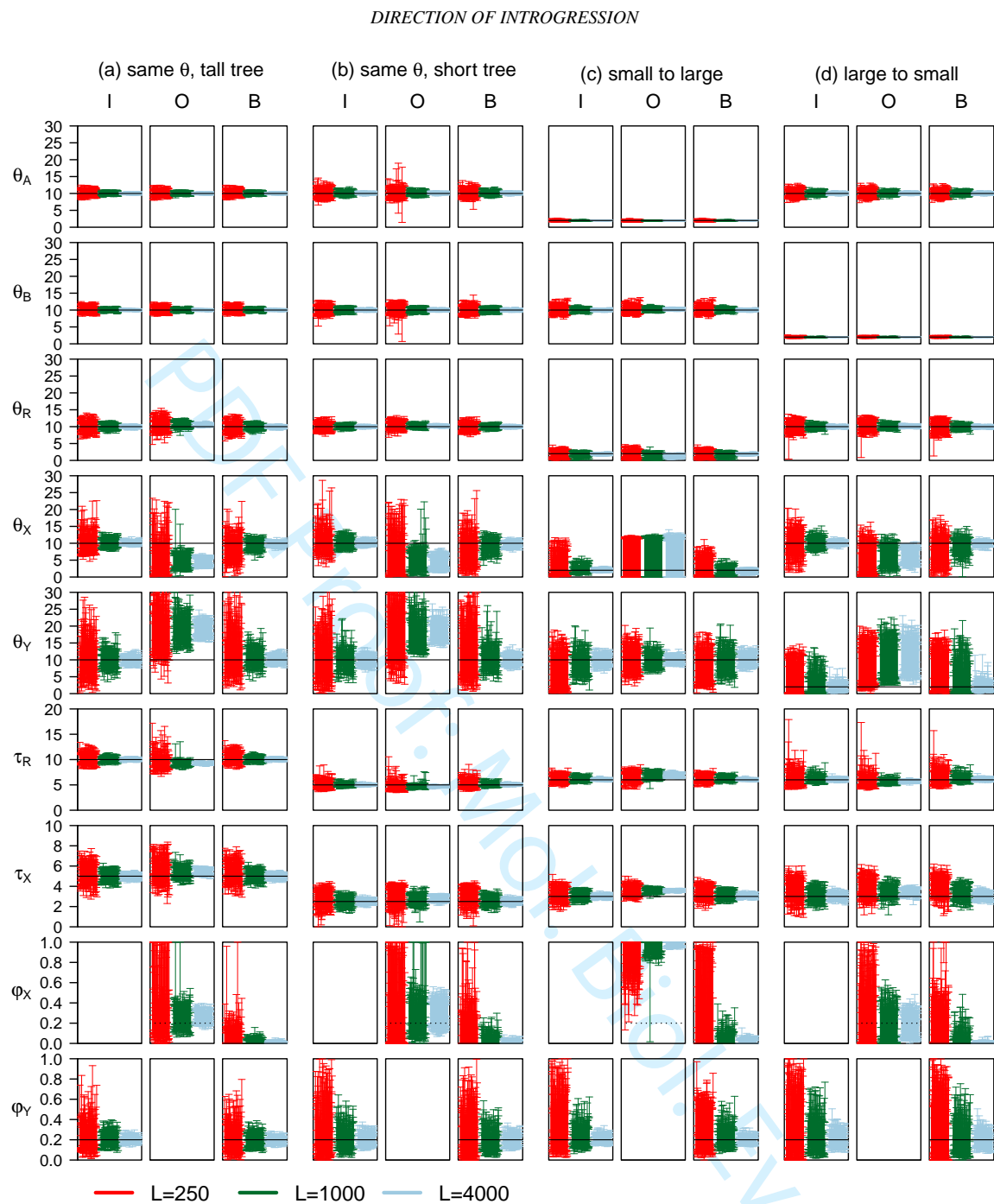


Figure 3: The 95% HPD CIs for parameters in 100 replicate datasets (each of L loci) simulated under model I and analyzed under models I, O, and B of figure 1a–c. Four sets of parameter values are used (cases a–d) (table S1). Parameters θ s and τ s are multiplied by 10^3 . Black solid lines indicate the true values. Dotted lines for φ_X in model O indicate the true value of φ_Y in model I.

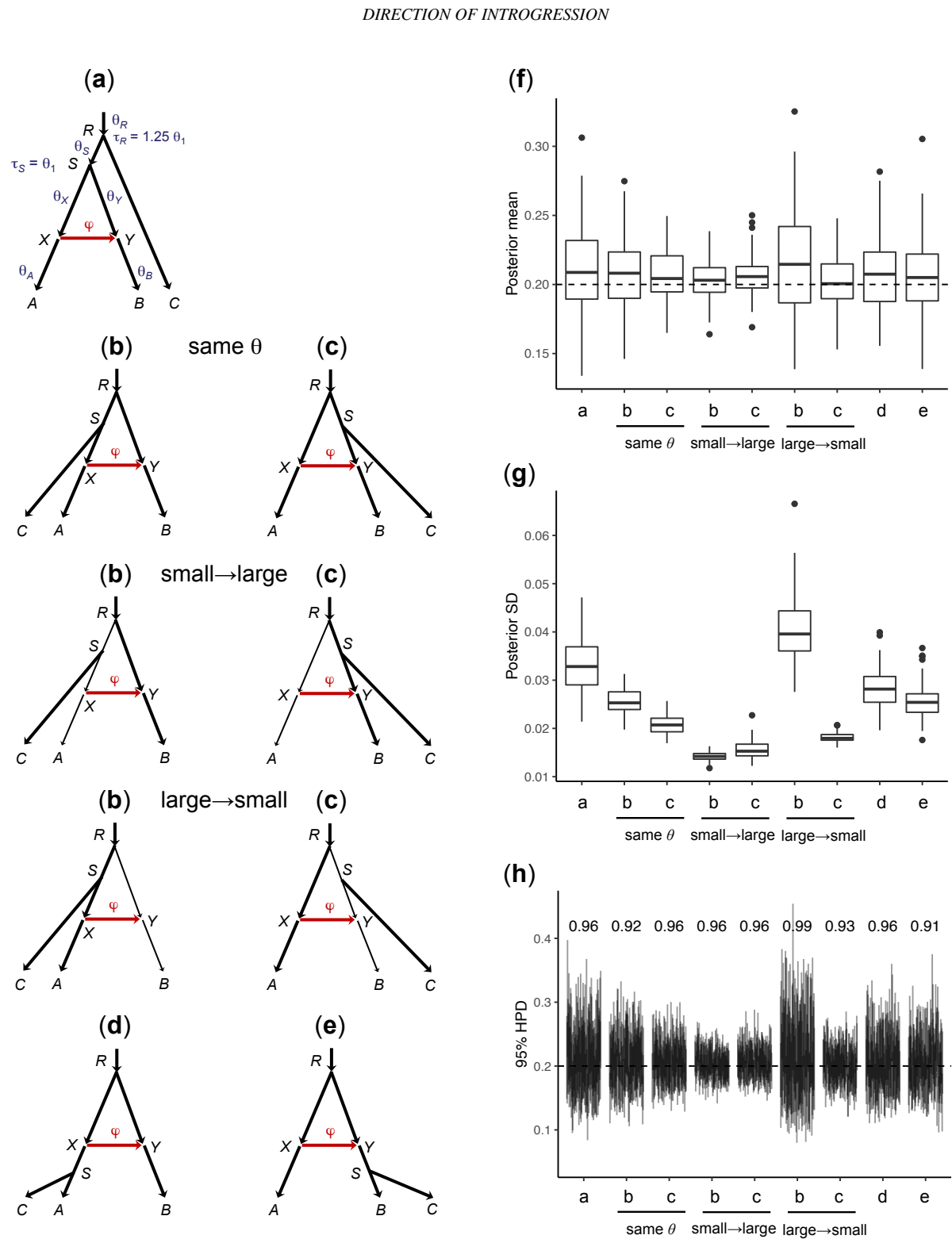


Figure 4: (a–e) MSci models for three species (A, B, C), with introgression from A to B, obtained by adding a third species C onto the two-species tree of figure 1a at five possible locations: (a) root population, (b, c) source and target populations before gene flow, and (d, e) source and target populations after gene flow. (f) Box plots of the posterior means for φ among 100 replicate datasets simulated under each of the five cases (a–e). The dashed line indicates the true value ($\varphi = 0.2$). (g) Box plots of the posterior SD for φ . (h) 95% HPD CIs for φ , with the CI coverage above the CI bars. See figure S3 for CIs for other parameters.

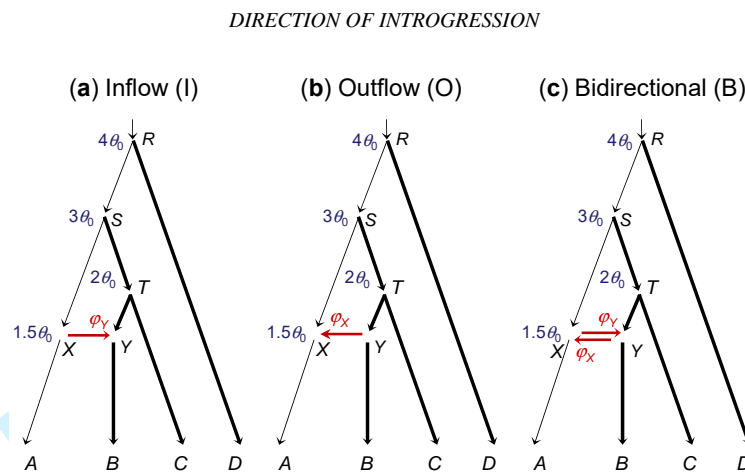


Figure 5: **(4s-trees)** Three MSci models for four species differing in introgression direction assumed to simulate and analyze data: **(a)** inflow from *A* to *B* (I); **(b)** outflow from *B* to *A* (O); and **(c)** bidirectional introgression between *A* and *B* (B). Divergence times used are shown next to the nodes: $\tau_R = 4\theta_0$, $\tau_S = 3\theta_0$, $\tau_T = 2\theta_0$, and $\tau_X = \tau_Y = 1.5\theta_0$, with population sizes $\theta_0 = 0.002$ for the thin branches and $\theta_1 = 0.01$ for the thick branches. We also used a setting in which all populations on the species tree have the same size, with $\theta_0 = \theta_1 = 0.01$. Introgression probabilities are $\varphi_X = \varphi_Y = 0.2$.

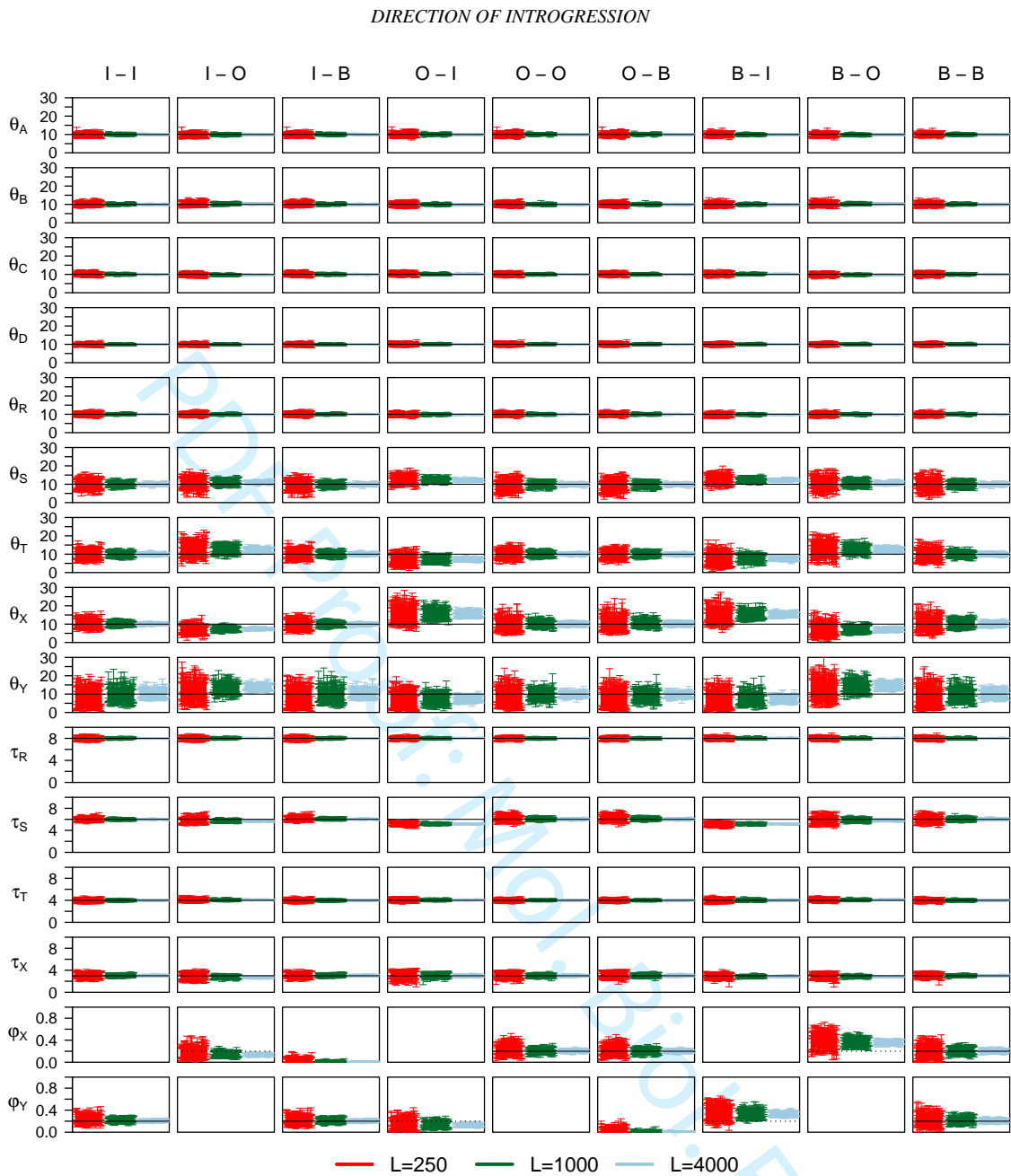


Figure 6: **(4s-same- θ)** The 95% HPD CIs for parameters in 100 replicate datasets simulated and analyzed under models I, O, and B of figure 5, with all species on the species tree having the same population size ($\theta_0 = \theta_1 = 0.01$) when the data are generated. The nine settings are specified in the simulation-analysis format; i.e., ‘I-O’ means that data were simulated under model I and analyzed under model O. Parameters θ and τ are multiplied by 10^3 . Horizontal solid lines indicate the true values. In the I-O setting, the dotted line for φ_X in model O indicates the true value of φ_Y in model I assumed in the simulation, while in the O-I setting, the dotted line for φ_Y indicates the true value of φ_X in the assumed O model. In the B-I and B-O settings, two introgression probabilities exist in the simulation model (φ_X, φ_Y) but only one is assumed in the analysis model, and the dotted line indicates its true value.

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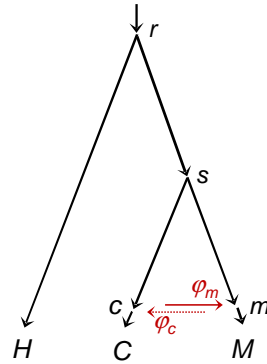


Figure 7: Species tree for *Heliconius hecale* (*H*), *H. cydno* (*C*), and *H. melpomene* (*M*), with introgression between *H. cydno* and *H. melpomene*, used to analyze genomic sequence data. Parameters in the MSci model include species divergence and introgression times ($\tau_r, \tau_s, \tau_c = \tau_m$), population sizes for branches on the species tree (e.g., θ_C for branch *C* and θ_c for branch *sc*), as well as introgression probabilities ($\varphi_m \equiv \varphi_{C \rightarrow M}$ and $\varphi_c \equiv \varphi_{M \rightarrow C}$). The data support the $C \rightarrow M$ introgression but not the $M \rightarrow C$ introgression, with $\varphi_m > 0$ and $\varphi_c \approx 0$ (tables 2 and S4, fig. S7).

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Table 1. Features of the data that are informative about parameters in the wrong model O when data are generated under model I with parameter $\Theta^{(I)}$ (fig. 1a)

Parameter in model O	Information in data
(a) Introgression time: $\hat{\tau}_X^{(O)} \approx \tau_X^{(I)}$	$\min\{t_{ab}\}$
(b) Species divergence time: $\hat{\tau}_R^{(O)} \approx \tau_R^{(I)}$	Discontinuities in $f(t_{aa}), f(t_{ab}), f(t_{bb})$
(c) Population sizes for extant species: $\hat{\theta}_A^{(O)} \approx \theta_A^{(I)}, \hat{\theta}_B^{(O)} \approx \theta_B^{(I)}$	$f(t_{aa}), f(t_{bb})$ over $(0, \tau_X)$
(d) Population sizes for ancestral species not involved in introgression: $\hat{\theta}_R^{(O)} \approx \theta_R^{(I)}$	$f(t_{aa}), f(t_{ab}), f(t_{bb})$ over (τ_X, ∞)
(e) Ancestral population size: $\hat{\theta}_X^{(O)} < \theta_X^{(I)}$	$f(t_{aa})$ over (τ_X, τ_R)
(f) Ancestral population size: $\hat{\theta}_Y^{(O)} > \theta_Y^{(I)}$	$f(t_{bb})$ over (τ_X, τ_R)
(g) Introgression probability: $\hat{\varphi}_X [1 - e^{-2\Delta\tau/\hat{\theta}_Y^{(O)}}] = \varphi_Y [1 - e^{-2\Delta\tau/\theta_X^{(I)}}]$ (eq. 1): $\hat{\varphi}_X > \varphi_Y$ if $\hat{\theta}_Y^{(O)} > \theta_X^{(I)}$, $\hat{\varphi}_X < \varphi_Y$ if $\hat{\theta}_Y^{(O)} < \theta_X^{(I)}$.	$f(t_{ab})$ over (τ_X, τ_R)

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Table 2. Posterior means and 95% HPD CIs for parameters in bPP analyses of two datasets of noncoding and coding loci on chromosome 1 from *Heliconius* butterflies (fig. 7) under four models with different introgression directions

	model \emptyset (no gene flow)	Model I ($C \rightarrow M$)	Model O ($M \rightarrow C$)	Model B ($C \rightleftharpoons M$)
Noncoding loci ($L = 5,341$ loci)				
θ_H	0.0131 (0.0127, 0.0136)	0.0134 (0.0129, 0.0139)	0.0134 (0.0129, 0.0138)	0.0134 (0.0129, 0.0139)
θ_C	0.0407 (0.0329, 0.0496)	0.0500 (0.0274, 0.0759)	0.0231 (0.0070, 0.0415)	0.0499 (0.0267, 0.0759)
θ_M	0.0026 (0.0021, 0.0031)	0.0003 (0.0002, 0.0005)	0.0001 (0.0000, 0.0002)	0.0003 (0.0002, 0.0005)
θ_r	0.0124 (0.0119, 0.0128)	0.0123 (0.0118, 0.0127)	0.0122 (0.0118, 0.0127)	0.0123 (0.0118, 0.0127)
θ_s	0.0343 (0.0328, 0.0358)	0.0152 (0.0141, 0.0162)	0.0185 (0.0175, 0.0194)	0.0152 (0.0141, 0.0162)
θ_c	n/a	0.0256 (0.0241, 0.0271)	0.0230 (0.0206, 0.0254)	0.0255 (0.0240, 0.0270)
θ_m	n/a	0.0188 (0.0162, 0.0214)	0.0294 (0.0262, 0.0327)	0.0189 (0.0164, 0.0215)
τ_r	0.0116 (0.0114, 0.0117)	0.0118 (0.0116, 0.0120)	0.0118 (0.0116, 0.0120)	0.0118 (0.0116, 0.0120)
τ_s	0.0010 (0.0008, 0.0012)	0.0068 (0.0064, 0.0072)	0.0051 (0.0048, 0.0053)	0.0068 (0.0064, 0.0071)
$\tau_c = \tau_m$	n/a	0.0001 (0.0001, 0.0002)	0.0000 (0.0000, 0.0001)	0.0001 (0.0001, 0.0002)
φ_c	n/a	n/a	0.1744 (0.1458, 0.2038)	0.0019 (0.0000, 0.0057)
φ_m	n/a	0.2830 (0.2565, 0.3090)	n/a	0.2802 (0.2530, 0.3067)
Coding loci ($L = 4,942$ loci)				
θ_H	0.0055 (0.0053, 0.0058)	0.0055 (0.0053, 0.0058)	0.0055 (0.0052, 0.0057)	0.0055 (0.0053, 0.0058)
θ_C	0.0054 (0.0048, 0.0060)	0.0361 (0.0203, 0.0545)	0.0307 (0.0133, 0.0513)	0.0363 (0.0204, 0.0553)
θ_M	0.0016 (0.0015, 0.0018)	0.0010 (0.0008, 0.0011)	0.0005 (0.0003, 0.0008)	0.0010 (0.0008, 0.0011)
θ_r	0.0092 (0.0088, 0.0096)	0.0092 (0.0088, 0.0096)	0.0094 (0.0090, 0.0098)	0.0092 (0.0088, 0.0096)
θ_s	0.0117 (0.0111, 0.0124)	0.0027 (0.0004, 0.0054)	0.0092 (0.0084, 0.0100)	0.0027 (0.0004, 0.0053)
θ_c	n/a	0.0059 (0.0055, 0.0063)	0.0044 (0.0032, 0.0055)	0.0058 (0.0053, 0.0062)
θ_m	n/a	0.0119 (0.0076, 0.0168)	0.0105 (0.0072, 0.0144)	0.0129 (0.0077, 0.0189)
τ_r	0.0049 (0.0047, 0.0050)	0.0049 (0.0047, 0.0050)	0.0048 (0.0047, 0.0050)	0.0049 (0.0047, 0.0050)
τ_s	0.0009 (0.0008, 0.0010)	0.0047 (0.0045, 0.0049)	0.0017 (0.0015, 0.0019)	0.0047 (0.0045, 0.0049)
$\tau_c = \tau_m$	n/a	0.0005 (0.0004, 0.0006)	0.0002 (0.0001, 0.0003)	0.0005 (0.0004, 0.0006)
φ_c	n/a	n/a	0.1360 (0.0783, 0.1959)	0.0073 (0.0000, 0.0194)
φ_m	n/a	0.5119 (0.4780, 0.5451)	n/a	0.5064 (0.4722, 0.5412)

Note.— Results for the other chromosomes are summarized in table S4 and figure S7.

DIRECTION OF INTROGRESSION

Table 3. Bayes factors for comparing four introgression models for the *Heliconius* datasets (fig. 7, table 2), calculated using thermodynamic integration with 32 or 64 Gaussian quadrature points and Savage-Dickey density ratio with threshold $\epsilon = 1\%$, 0.1% , or 0.01%

B_{ij} (null hypothesis tested, H_0)	Thermodynamic integration		Savage-Dickey density ratio		
	32 points	64 points	$\epsilon = 1\%$	$\epsilon = 0.1\%$	$\epsilon = 0.01\%$
Noncoding loci ($L = 5,341$ loci)					
$B_{I\emptyset}$ ($H_0: \varphi_{C \rightarrow M} = 0$)	$e^{1087.1}$	$e^{1082.5}$	∞	∞	∞
$B_{O\emptyset}$ ($H_0: \varphi_{M \rightarrow C} = 0$)	$e^{946.9}$	$e^{904.9}$	∞	∞	∞
B_{BI} ($H_0: \varphi_{M \rightarrow C} = 0$)	$e^{-5.6}$	$e^{-9.9}$	0.0101	0.0025	0.0020
B_{BO} ($H_0: \varphi_{C \rightarrow M} = 0$)	$e^{134.6}$	$e^{167.8}$	∞	∞	∞
B_{IO} ($H_0: \varphi_{C \rightarrow M} = 0$ vs. $\varphi_{M \rightarrow C} = 0$)	$e^{140.2}$	$e^{177.6}$	n/a	n/a	n/a
$B_{B\emptyset}$ ($H_0: \varphi_{C \rightarrow M} = 0$ and $\varphi_{M \rightarrow C} = 0$)	$e^{1081.6}$	$e^{1072.6}$	∞	∞	∞
Coding loci ($L = 4,942$ loci)					
$B_{I\emptyset}$ ($H_0: \varphi_{C \rightarrow M} = 0$)	$e^{359.9}$	$e^{358.5}$	∞	∞	∞
$B_{O\emptyset}$ ($H_0: \varphi_{M \rightarrow C} = 0$)	$e^{128.0}$	$e^{147.6}$	∞	∞	∞
B_{BI} ($H_0: \varphi_{M \rightarrow C} = 0$)	$e^{-13.0}$	$e^{-8.6}$	0.0136	0.0090	0.0073
B_{BO} ($H_0: \varphi_{C \rightarrow M} = 0$)	$e^{218.9}$	$e^{202.3}$	∞	∞	∞
B_{IO} ($H_0: \varphi_{C \rightarrow M} = 0$ vs. $\varphi_{M \rightarrow C} = 0$)	$e^{231.9}$	$e^{210.9}$	n/a	n/a	n/a
$B_{B\emptyset}$ ($H_0: \varphi_{C \rightarrow M} = 0$ and $\varphi_{M \rightarrow C} = 0$)	$e^{346.8}$	$e^{349.9}$	∞	∞	∞

Note.— The four models are (\emptyset) MSC with no gene flow, (I) $C \rightarrow M$ introgression (I), (O) $M \rightarrow C$ introgression, and (B) $C \rightleftharpoons M$ bidirectional introgression (table 2). Bayes factor B_{ij} represents the evidence in favour of model i against model j . We use a cut-off of 1%, so that $B_{ij} > 100$ means strong support for model i and rejection of model j , $B_{ij} < 0.01$ means strong support for model j and rejection of model i , while $0.01 < B_{ij} < 100$ means no strong preference for either model. The approach based on Savage-Dickey density ratio is inapplicable for B_{IO} as models I and O are not nested. Also it produces $B = \infty$ if all values of φ in the MCMC sample are $> \epsilon$. Results for the other chromosomes are shown in table S5.