

1 **Phylogenomic approaches to detecting and characterizing introgression**

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9 **Abstract**

10 Phylogenomics has revealed the remarkable frequency with which introgression occurs across  
11 the tree of life. These discoveries have been enabled by the rapid growth of methods designed to  
12 detect and characterize introgression from whole-genome sequencing data. A large class of  
13 phylogenomic methods makes use of data from one sample per species to infer introgression  
14 based on expectations from the multispecies coalescent. These methods range from simple tests,  
15 such as the *D*-statistic, to model-based approaches for inferring phylogenetic networks. Here, we  
16 provide a detailed overview of the various signals that different modes of introgression are  
17 expected leave in the genome, and how current methods are designed to detect them. We discuss  
18 the strengths and pitfalls of these approaches and identify areas for future development, using a  
19 small simulation study to highlight the different signals of introgression and the power of each  
20 method to detect them. We conclude with a discussion of how to visualize and interpret the  
21 results of introgression analyses.

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## 42 **Introduction**

43 The potential for hybridization and subsequent backcrossing between lineages—also known as  
44 introgression—has long been understood (Heiser 1949, Heiser 1973, Rieseberg and Wendel  
45 1993, Dowling and Secor 1997). However, until genome sequencing became widely available to  
46 biologists, it was difficult to quantify patterns of introgression effectively and reliably. As a  
47 result, introgression’s role in evolution was under-appreciated, especially in animal systems. In  
48 part precipitated by the discovery of introgression between archaic human populations (Green et  
49 al. 2010, Huerta-Sanchez et al. 2014), the past decade has seen an explosive increase in the rate  
50 of discovery of reticulate evolution across the tree of life (Mallet et al. 2016, Taylor and Larson  
51 2019). Although great efforts have been made in recent years to synthesize the biological  
52 implications of these discoveries (Hedrick 2013, Ellstrand et al. 2013, Harrison and Larson 2014,  
53 Racimo et al. 2015, Ottenburghs et al. 2017, Suarez-Gonzalez et al. 2018), comparatively little  
54 synthesis has been provided on the accompanying growth in methods used to detect and  
55 characterize introgression.

56 The information that can be gleaned from genomic data about introgression depends on both the  
57 number of sampled species and the number of sampled individuals. Methods with only two  
58 species or populations depend on sampling multiple individuals within at least one of them.  
59 Patterns of nucleotide variation among individuals and across loci can then be used to make  
60 inferences about introgression (e.g. Wakeley and Hey 1997, Nielsen and Wakeley 2001, Joly et  
61 al. 2009, Lohse and Frantz 2014, Rosenzweig et al. 2016, Schrider et al. 2018). Because less  
62 information is available about phylogenetic relationships, these methods often rely on the  
63 assumption that all sequenced loci are evolving neutrally or that all loci have the same rate of  
64 nucleotide substitution (or both). For these reasons such methods are more prone to model  
65 violations, such as the heterogeneous effects of background selection across loci (Roux et al.  
66 2016). Despite these limitations, population-genetic methods are one of the few approaches that  
67 can infer gene flow between pairs of sister taxa (see Hahn 2018 for more details).

68 When there is data for a rooted triplet of species—or an unrooted quartet—it becomes possible to  
69 construct more powerful tests for introgression using genome-scale datasets. Importantly, this  
70 can be done using only a single sample per species and without assumptions about neutrality.  
71 The robustness to non-neutral processes occurs because much of the genealogical signal of  
72 introgression is not mimicked by selection (Przeworski et al. 1999, Williamson and Orive 2002,  
73 Vanderpool et al. 2020). This class of “phylogenomic” methods is largely based on one sample  
74 per species, but also includes methods based on multiple samples. One-sample methods include  
75 the *D* statistic (also known as the ABBA-BABA test; Green et al. 2010, Durand et al. 2011), its  
76 numerous analogs and extensions (see below), methods based on pairwise sequence divergence  
77 such as the *D*<sub>3</sub> statistic (Hahn and Hibbins 2019), and phylogenetic network approaches such as  
78 those implemented in *PhyloNet* (Than et al. 2008, Wen et al. 2018), *SNaQ* (Solís-Lemus and Ané  
79 2016), and *SpeciesNetwork* (Zhang et al. 2018). When multiple individuals are sampled from  
80 very closely related species or populations, additional power may be gained by measuring the

81 deviation from covariances in allele frequency expected under strictly treelike evolution (Reich  
82 et al. 2009, Patterson et al. 2012, Pickrell and Pritchard 2012, Peter 2016).

83 In this review, we focus on phylogenomic methods for studying introgression, most of which are  
84 based on the multispecies coalescent model. We provide a detailed overview of the signals that  
85 various introgression scenarios are expected to leave in the genome, and the methods that are  
86 designed to detect these signals. We discuss common misuses and misinterpretations of these  
87 methods, as well as providing recommendations for best-use practices. Finally, we present  
88 results from a small simulation study conducted across different introgression scenarios to  
89 highlight the advantages and limitations of currently available methods. Based on these results,  
90 we identify areas for future theoretical and methodological advancement.

## 91 **Biological processes that generate gene tree heterogeneity**

92 We begin our discussion of phylogenomic methods with the simplest possible sampling scheme:  
93 genomic data from a single sampled haploid individual from each of three focal species and an  
94 outgroup. By “genomic data” we mean data sampled from many loci across the genome, with the  
95 standard assumption of no intra-locus recombination and free inter-locus recombination. This  
96 data structure will hereafter be referred to as a quartet or rooted triplet. For three ingroup species,  
97  $P1$ ,  $P2$ , and  $P3$ , and an outgroup species,  $O$ , there are three possible tree topologies describing  
98 how they can be related:  $((P1,P2),P3),O$ ,  $((P2,P3),P1),O$ , or  $((P1,P3),P2),O$  (Figure 1). In  
99 addition to a single phylogeny describing the evolutionary history of the quartet, trees can be  
100 constructed for each individual locus. The frequencies of each topology across loci are referred  
101 to as gene tree frequencies, even when they do not come from protein-coding genes. This  
102 heterogeneity in both the topology and branch lengths of gene trees is caused by two different  
103 biological processes: incomplete lineage sorting and introgression. Below we describe the  
104 expected effects of both processes in order to understand how tests for introgression work.

### 105 ***Incomplete lineage sorting as a null hypothesis for tests of introgression***

106 The phenomenon of incomplete lineage sorting (ILS), in which two or more lineages fail to  
107 coalesce with each other before reaching an ancestral population (looking backwards in time),  
108 can result in individual gene trees that are discordant with the species history (Figure 1).  
109 Phylogenomic methods must account for this phenomenon to make accurate inferences about  
110 introgression. Discordant gene trees occur because, when ILS occurs, it becomes possible for the  
111 order of coalescent events to differ from the order of splits in the species phylogeny (Figure 1,  
112 top right panel). Gene tree discordance due to ILS is very common in modern phylogenomic  
113 datasets (e.g. Pollard et al. 2006, Fontaine et al. 2015, Pease et al. 2016, Novikova et al. 2016,  
114 Copetti et al. 2017, Wu et al. 2018a; Edelman et al. 2019) and can arise within phylogenies that  
115 contain no introgression events. Because both ILS and introgression can generate many of the  
116 same genealogical patterns, it is essential to incorporate ILS into the null hypothesis of tests for  
117 introgression.

118 Fortunately, the parameters mostly likely to influence the probability of ILS—time between  
119 speciation events and ancestral population size—are well understood from the multispecies  
120 coalescent (MSC) model (Hudson 1983, Tajima 1983, Pamilo and Nei 1988). For a rooted

121 triplet, the probability that the two sister lineages (e.g.  $P1$  and  $P2$  in Figure 1) coalesce in their  
122 most recent common ancestral population is given by the formula  $1 - e^{-\tau}$ , where  $\tau$  is the length  
123 of this internal branch in units of  $2N$  generations (sometimes referred to as "coalescent units").  
124 Conversely, the probability of ILS (i.e. that they do not coalesce) is  $e^{-\tau}$ . If ILS occurs, all three  
125 lineages ( $P1$ ,  $P2$ , and  $P3$ ) enter their joint ancestral population. Within this population the  
126 coalescent events happen at random, such that lineages leading to each pair of species have a  $1/3$   
127 chance of coalescing first. This means that the two discordant gene tree topologies are expected  
128 to be equal in frequency (Figure 1, top right), with probabilities of  $1/3 e^{-\tau}$  each. In addition, the  
129 concordant tree topology can be produced either by lineage sorting with probability  $1 - e^{-\tau}$  or  
130 incomplete lineage sorting with probability  $1/3 e^{-\tau}$  (Figure 1, top left). This guarantees that the  
131 concordant tree topology will always be at least as frequent as the two discordant trees (Figure 1,  
132 top row). These expectations under ILS form the null hypothesis for tests of introgression based  
133 on gene tree frequencies.

134 In addition to gene tree frequencies, ILS affects expected coalescence times, and therefore  
135 sequence divergence, between pairs of species. In any population, the expected times to  
136 coalescence depends on how many lineages are present (Kingman 1982, Hudson 1983, Tajima  
137 1983). If three lineages are present, the first coalescence is expected to occur  $2/3 N$  generations  
138 in the past. After this first coalescence—or if only two lineages were present to begin with—the  
139 next coalescence is expected a further  $2N$  generations in the past. These expectations are equally  
140 applicable to current populations as to ancestral populations, but coalescence cannot occur until  
141 the lineages under consideration are in a common population. Therefore, expected coalescence  
142 times between species always have the time of speciation included as a constant, no matter how  
143 far back lineage-splitting occurred (Gillespie and Langley 1979).

144 For example, the time to coalescence between species  $P1$  and  $P2$  in Figure 1 is expected to be  $2N$   
145 generations prior to their speciation event. If this coalescent event happens in their most recent  
146 common ancestral population (i.e. lineage sorting), then the next coalescent event occurs  
147 between the resulting single lineage and the lineage leading to  $P3$  in the common ancestral  
148 population of all three species (Figure 1, bottom row). This event is again  $2N$  generations prior to  
149 the speciation event between  $P3$  and the common ancestor of  $P1+P2$ . If ILS occurs, then the first  
150 coalescence (regardless of which lineages are involved) occurs  $2/3 N$  generations prior to this  
151 same speciation event, and the second coalescence  $2N$  generations before this. Note that, if we  
152 condition on lineage sorting having occurred, the expected coalescence times become slightly  
153 more complicated (see Mendes and Hahn 2018, Hibbins and Hahn 2019 for exact expectations)

154 The two pairs of non-sister lineages in a rooted triplet ( $P1$  and  $P3$  or  $P2$  and  $P3$  in Figure 1) can  
155 coalesce at one of two times, depending on whether they are the first or second pair to coalesce  
156 in a gene tree (there can only be a discordant topology if they are the first to coalesce). Owing to  
157 the symmetry of gene tree topology shapes and frequencies, these times are equivalent across  
158 loci, leading to the null expectation under ILS that genome-wide divergence between both pairs  
159 of non-sister taxa should be equal (Figure 1, bottom row). Finally, each of these coalescence  
160 times is expected to follow a unimodal exponential distribution under ILS alone (Hudson 1983,  
161 Tajima 1983).

162 *The effects of introgression on gene trees*

163 Introgression between two lineages occurs when an initial hybridization event is followed by  
164 back-crossing into one or both of the parental lineages. Hybridization itself—the creation of a  
165 hybrid individual—is generally not sufficient to be called introgression, though polyploid or  
166 homoploid hybrid species will be identified by many of the same tests described here (e.g. Meng  
167 and Kubatko 2009; Blischak et al. 2018; Folk et al. 2018). Similarly, horizontal gene transfer  
168 will also generate discordant gene trees, but introgression is generally distinguished from this  
169 process by the requirement that there be mating between the hybridizing lineages in order to be  
170 considered introgression. In addition, the mating requirement means that the hybridizing species  
171 are closely related enough such that the tree topologies produced by introgression will likely be  
172 the same as those produced by ILS. Horizontal gene transfer, on the other hand, can produce  
173 highly discordant topologies that can only be produced by the interspecific exchange of genetic  
174 material (e.g. Knowles et al. 2018).

175 There are a large number of different introgression scenarios, each with a different effect on the  
176 underlying gene trees. While there are well-developed mathematical tools that describe the  
177 effects of introgression on gene tree topologies (e.g. the multispecies network coalescent;  
178 reviewed in Degnan 2018, Elworth et al. 2019), we generally do not need the predictions from  
179 these models to test for the presence of introgression (with some exceptions discussed below).  
180 Instead, because our tests are often simply looking for a rejection of the ILS-only model, a  
181 general understanding of the key outcomes of introgression will be sufficient. Figure 2  
182 summarizes the scenarios involving introgression that are most commonly encountered.

183 As a first key distinction, introgression can occur either between sister lineages (events 1 and 2  
184 in Figure 2A) or non-sister lineages (events 3, 4, and 5 in Figure 2A). As a general rule,  
185 introgression between sister lineages should increase the proportion of concordant gene trees  
186 relative to the case of ILS alone. To see why this is, consider introgression event 1 in Figure 2:  
187 gene flow after speciation between *P1* and *P2* effectively increases  $\tau$ , the length of the internal  
188 branch separating these two lineages from their common ancestor with *P3*. Loci with an  
189 introgressed history therefore have a reduced probability of ILS because of the increased time for  
190 them to coalesce. While there are some exceptions to this rule—all of which involve  
191 introgression between sister lineages on an internal branch of the species tree (i.e. event 2 in  
192 Figure 2; Solis-Lemus et al. 2016, Long and Kubatko 2018, Jiao and Yang 2020)—in no cases  
193 should gene flow between sister lineages result in one discordant topology becoming more  
194 common than the other discordant topology.

195 Because an increase in concordant topologies can also be generated under an ILS-only model  
196 with a longer internal branch in the species tree, gene tree frequencies alone cannot tell us  
197 whether introgression has occurred between sister lineages. Note, however, that loci with a  
198 history of introgression can have a different distribution of branch lengths in this scenario than  
199 expected under ILS alone: the coalescence times are more recent than expected under ILS for  
200 either event 1 or 2 (Figure 2B). Our ability to determine whether the distribution of branch  
201 lengths is due to a history of introgression partly depends on whether gene flow is continuously  
202 occurring after speciation or occurs as a single pulse of hybridization and backcrossing at a

203 period considerably after speciation: pulses of introgression following secondary contact  
204 between species will almost always be easier to detect (see section on "*Detecting introgression*  
205 *using coalescence times*"). Using only a single sample from each species, we also cannot  
206 determine the direction of gene flow between sister lineages; this is why we have drawn events 1  
207 and 2 as bidirectional introgression. In order to make this determination between sister species  
208 we must use population genetic methods (e.g. Schrider et al. 2018).

209 When introgression occurs between non-sister lineages (events 3, 4, and 5 in Figure 2A) then one  
210 discordant tree topology can become more common than the other. The resulting asymmetry in  
211 discordant tree topologies is one of the clearest signals of introgression. In both events 3 and 4  
212 we expect loci that have introgressed to be more likely to have a gene tree topology placing  $P2$   
213 and  $P3$  sister to one another:  $((P2,P3),P1)$  (Figure 2C). While not all loci following this  
214 introgression history will have this discordant topology, the extended period of shared history  
215 between  $P2$  and  $P3$  makes it more likely for these lineages to coalesce. In general, the strength of  
216 the asymmetry in discordant topologies will depend on the net rate, timing, and direction of  
217 introgression (Durand et al. 2011; Martin et al. 2015; Zheng and Janke 2018), as well as the  
218 absence of introgression between the other non-sister pair (in which case the other discordant  
219 topology would also go up in frequency). Although the same discordant topology will be  
220 produced in excess by events 3 and 4 (Figure 2C), note that the resulting branch lengths will  
221 differ on average between the two. This difference makes it possible to determine the main  
222 direction of introgression between non-sister taxa (see below). Although we have drawn gene  
223 flow as unidirectional to highlight the fact that this distinction can be made, bidirectional gene  
224 flow between these lineages is equally biologically plausible.

225 Finally, event 5 depicts an introgression event involving an unsampled ("ghost") lineage. For  
226 many of the signals of introgression discussed here, the sampled lineages included in a study  
227 may not be the ones that actually hybridized. Whether species go unsampled because they are  
228 extinct or simply unavailable, non-sister ghost lineages that act as donors in introgression events  
229 will often generate detectable patterns of gene flow. These patterns can result in misleading  
230 inferences about the lineages involved in gene flow and the direction of gene flow, and should  
231 therefore always be kept in mind; we include introgression from ghost lineages in our simulation  
232 study below to demonstrate some of these effects. Ghost lineages that are either the recipients of  
233 gene flow or are sister to sampled taxa are much less likely to leave any signal of introgression.  
234 For similar reasons, the sister lineages shown in Figure 2 do not need to be one another's most  
235 closely related species in nature; what is important is whether they are sister (or non-sister)  
236 among sampled species.

## 237 **Detecting introgression using gene tree frequencies**

### 238 *The D statistic*

239 Perhaps the most widely used method for inferring introgression is the  $D$  statistic, or—perhaps  
240 because there are already so many  $D$ 's in use—what is commonly referred to as the ABBA-  
241 BABA test. This test was originally formulated to test for evidence of gene flow between

242 Neanderthals and archaic humans (Green et al. 2010, Durand et al. 2011), and is based on the  
243 effect of introgression between non-sister taxa on gene tree frequencies.

244 The statistic counts the occurrence of two configurations of shared derived alleles across three  
245 species and an outgroup. Assuming the species tree  $((P1,P2),P3)O$ , and denoting the ancestral  
246 allele as "A" and the derived allele as "B," there are two phylogenetically informative patterns of  
247 discordant sites. The pattern "ABBA" represents sites where  $P2$  and  $P3$  share a derived allele,  
248 while  $P1$  and the outgroup have the ancestral allele. The pattern "BABA" represents sites where  
249  $P1$  and  $P3$  share a derived allele, to the exclusion of  $P2$  and the outgroup (Figure 3). For clarity,  
250 note that sites supporting the species topology would have the pattern BBAA; however, these are  
251 not used in this statistic.

252 The  $D$  statistic assumes an infinite sites model, meaning that the two discordant site patterns can  
253 only arise via single mutations on the internal branches of discordant gene trees (Figure 3, blue  
254 dots/branches). Under this assumption, the frequencies of ABBA and BABA site patterns are  
255 expected to reflect the frequencies of underlying gene trees. If the number of ABBA and BABA  
256 sites differ significantly, then an asymmetry in gene tree topologies is inferred, with  
257 introgression occurring between the species sharing the derived state more frequently. Figure 3  
258 depicts the scenario when the site pattern ABBA is more common, implying introgression  
259 between  $P2$  and  $P3$ .

260 To make it comparable across studies, the value of the  $D$  statistic is typically reported after  
261 normalization using the sum of ABBA and BABA pattern counts, giving the following formula:

$$262 \quad D = \frac{ABBA - BABA}{ABBA + BABA}$$

263 where ABBA and BABA represent the number of sites of each type. This statistic has an  
264 expected value of  $D = 0$  if there is no gene flow. When used as a whole-genome test of  
265 introgression between non-sister taxa, the  $D$ -statistic is robust under many different scenarios  
266 (Zheng and Janke 2018, Kong and Kubatko 2021), but can be affected by certain forms of  
267 ancestral population structure (Slatkin and Pollack 2008, Durand et al. 2011, Lohse and Frantz  
268 2014) (see section entitled "*Distinguishing introgression from ancestral population structure*"  
269 for more discussion of this issue).

270 Despite the widespread popularity and relative robustness of  $D$ , there are several important  
271 considerations and limitations to its use, some of which are often overlooked. The first of these  
272 concerns how to properly test the null hypothesis that  $D = 0$ . The expected site pattern counts of  
273 the  $D$ -statistic can easily be calculated, so it may be tempting to use a parametric test for  
274 differences. However, such tests assume that individual observations represent independent  
275 samples: this assumption is violated because closely spaced sites often share the same underlying  
276 local genealogy, making them non-independent. The pseudoreplication that results from treating  
277 all sites independently leads to inaccurate  $p$ -values. The solution to this issue is to use a block-  
278 bootstrap (or block-jackknife) approach to estimate the sample variance and then to calculate the  
279  $p$ -value (Green et al. 2010). This approach correctly accounts for correlations within blocks of  
280 adjacent sites.



281 Although formulated as a single genome-wide test, there are cases where the  $D$ -statistic has been  
282 applied to look for introgression in smaller genomic windows (e.g. Kronforst et al. 2013, Zhang  
283 et al. 2016, Wu et al. 2018b, Grau-Bové et al. 2020). However, the genome-wide expectation  
284 under ILS alone that  $D = 0$  does not hold true for smaller genomic windows. Since a single non-  
285 recombining locus contains a single genealogy by definition, it is only capable of generating one  
286 phylogenetically informative biallelic site pattern (again assuming an infinite sites mutation  
287 model). The consequence is that the value of  $D$  at a single locus can only be +1, 0, or -1,  
288 depending on the local genealogy (i.e. only ABBA, BBAA, or BABA). Therefore, even in ILS-  
289 only scenarios, there will be regions of the genome with extreme values of  $D$ , either positive or  
290 negative. This situation is more likely to occur in regions of low recombination, as in these  
291 regions even large genomic windows may only contain a small number of independent  
292 genealogies. Highlighting this problem, Martin et al. (2015) found that the variance of  $D$  is  
293 inflated in regions of low recombination, resulting in an excess of false positives if tests were to  
294 be performed on a per-window basis. Similar caution is warranted when applying  $D$  to  
295 inversions, as the entire inversion can act as a single locus (cf. Fuller et al. 2018). For these  
296 reasons, while it may be informative to plot the value of the  $D$  statistic along chromosomes, tests  
297 using  $D$  should be applied only to whole genomes, or at least to genomic regions that are  
298 sufficiently large to guarantee sampling a large number of underlying genealogies.

299 Finally, the  $D$ -statistic does not provide any information about introgression other than its  
300 presence or absence. While its value does increase with the rate of introgression, it is not a good  
301 estimator of this quantity, tending to greatly overestimate the true value (Martin et al. 2015,  
302 Hamlin et al. 2020). In addition, the sign of  $D$  is sometimes interpreted as providing information  
303 on the direction of introgression, though it can only identify which taxa are involved, and not the  
304 donor and recipient populations. For example, a significant  $D$  statistic implying introgression  
305 between  $P2$  and  $P3$  could involve the  $P3 \rightarrow P2$  direction, the  $P2 \rightarrow P3$  direction, or some  
306 combination of the two. Overall, the  $D$  statistic is a very reliable genome-wide test for  
307 introgression, but alternative methods are needed to infer more details about any detected  
308 introgression events.

### 309 ***Inferring the rate and direction of introgression using derived allele counts***

310 Many researchers are interested not only in the presence or absence of introgression, but in  
311 quantifying its magnitude. Methods for inferring introgression can often be used to estimate its  
312 “rate,” which can generally be taken to mean one of two things. In the context of phylogenomic  
313 approaches and phylogenetic networks, the rate refers to the proportion of the genome that  
314 originates from a history of introgression. This is also sometimes referred to as the “inheritance  
315 probability” or “admixture proportion.” Alternatively, in the isolation-with-migration (IM)  
316 framework, the rate refers to the movement of migrant individuals over continuous time  
317 (Wakeley and Hey 1998, Nielsen and Wakeley 2001). In this and following sections, we will  
318 take the “rate” to have the former definition.

319 The degree of asymmetry in discordant gene tree topologies contains information about the  
320 proportion of introgressed loci across the genome. However, simply using the  $D$  statistic does not  
321 provide an unbiased estimation of the rate (Martin et al. 2015, Hamlin et al. 2020). A recently

322 proposed extension of  $D$  called  $D_p$  makes one simple addition that improves the estimate of the  
323 proportion of introgressed loci. The statistic adds the counts of BBAA sites to the denominator to  
324 form:

$$325 \quad D_p = \left| \frac{ABBA - BABA}{BBAA + ABBA + BABA} \right|$$

326 Taking the degree of asymmetry as a fraction of the total number of phylogenetically informative  
327 biallelic sites brings  $D_p$  conceptually closer to estimating a genome-wide introgression  
328 proportion. The statistic tends to slightly underestimate the true rate of introgression—and its  
329 accuracy is affected by the direction of introgression—but it scales linearly with the rate of  
330 introgression and has better precision for lower true amounts of introgression (Hamlin et al.  
331 2020).

332 In an alternative approach, the observed value of an introgression test statistic is compared to the  
333 value that would be expected under a scenario where the entire genome was introgressed. The  
334  $F_4$ -ratio or  $\alpha$  statistic (Green et al. 2010, Patterson et al. 2012, Peter 2016) makes this comparison  
335 by taking the ratio of two  $F_4$  statistics (a genome-wide test for introgression based on allele  
336 frequencies). The  $\alpha$  statistic requires data from five samples and assumes an admixed population  
337 with two parent populations. *HyDe* (Blischak et al. 2018, Kubatko and Chifman 2019) estimates  
338 the rate in a similar way under a hybrid speciation scenario using linear combinations of derived  
339 site patterns. The assumptions of these methods are somewhat restrictive and are likely not  
340 reflective of the majority of introgression in nature (Schumer et al. 2014). However, *HyDe* gives  
341 highly accurate estimates of the rate of introgression when its assumptions about hybridization  
342 are met, and still provides reasonable estimates for the rate when these assumptions are violated  
343 (Kong and Kubatko 2021).

344 The  $f_d$  statistic of Martin et al. (2015) also takes the ratio of two  $D$ -statistics. However, by  
345 making the assumption that allele frequencies would be completely homogenized in a complete  
346 introgression scenario,  $f_d$  can be applied to quartets rather than requiring an additional sample.  
347 Like  $D_p$ ,  $f_d$  is sensitive to the direction of introgression because it estimates the proportion of the  
348 genome that came from the donor population during introgression. The  $f_d$  statistic somewhat  
349 overcomes this issue by assuming that the population with the higher derived allele frequency is  
350 the donor at each site. Nonetheless,  $f_d$  tends to underestimate the proportion of introgressed loci  
351 when  $P_2$  is the donor population.

352 Unless additional assumptions are made, there is not enough information contained in the  
353 frequency of gene tree topologies (i.e. site pattern counts) alone to estimate the direction of  
354 introgression in a quartet or rooted triplet. However, if a sample is obtained from a fifth species  
355 (Eaton and Ree 2013, Pease and Hahn 2015) or if polymorphism data is available for the quartet  
356 (Martin and Amos 2020), then it is possible to infer the direction of introgression. The  
357 “partitioned  $D$ -statistics” of Eaton and Ree (2013) were the first attempt to infer the direction of  
358 introgression in a five-taxon phylogeny. Unfortunately, redundant site pattern counts make the  
359 results of this directionality test uninterpretable. The  $D_{\text{FOIL}}$  method of Pease and Hahn (2015)  
360 resolves this problem by setting up a system of four  $D$  statistics, explicitly testing each of the 16

361 possible introgression events and directions.  $D_{\text{FOIL}}$  assumes that the 5-taxon phylogeny is  
362 symmetric, with two pairs of sister species. In this particular configuration of species it becomes  
363 possible to polarize introgression events because the direction of introgression affects  
364 relationships between the donor and both the recipient species and its sister taxon. Unfortunately,  
365  $D_{\text{FOIL}}$  does not work if the species tree is an asymmetric, or "caterpillar," tree.

366 Martin and Amos (2020) showed that information about the rate, direction, and timing of  
367 introgression in a quartet becomes available using site patterns if multiple individuals are  
368 sampled per lineage. Their approach, called the " $D$  frequency spectrum" or  $D_{\text{FS}}$  for short,  
369 estimates the  $D$  statistic in each bin of the joint derived allele frequency spectrum constructed for  
370 the two sister taxa in a quartet. The shape of the  $D_{\text{FS}}$  is expected to be affected by the direction of  
371 introgression. If one of the sister taxa is the recipient, then the spectrum is left-skewed, as the  
372 signal of introgression will be enriched among low-frequency alleles. In contrast, if a sister  
373 lineage is the donor and the non-sister lineage is the recipient, the spectrum is expected to be flat,  
374 because the frequency spectrum of the non-sister lineage is not used to construct the  $D_{\text{FS}}$ . The  
375 degree of left-skewness is affected by the timing of introgression, while the rate of introgression  
376 affects the magnitude of the  $D$ -statistic across bins. The shape of the  $D_{\text{FS}}$  is also affected by  
377 demographic history and changes under more complex introgression scenarios, so it will  
378 typically be necessary to perform simulations to explicitly test different introgression scenarios  
379 with this approach (Martin and Amos 2020).

### 380 *Inferring introgression events from reconstructed gene trees*

381 While methods based on site patterns and allele frequencies can be powerful, there are also  
382 fundamental limitations to the kinds of data they can be applied to. First, as mentioned earlier, a  
383 key assumption of the  $D$  statistic is an infinite sites model of mutation. When applied to closely  
384 related, extant species, this assumption is likely to hold. However, with increasing divergence it  
385 becomes more likely that ABBA and BABA site patterns can accumulate due to convergent  
386 substitutions. For this reason, site patterns are generally not a reliable way to test for  
387 introgression between more distantly related extant species, or along branches deeper in a species  
388 tree. Second, as the number of sampled species increases, the number of possible trees and  
389 quartets increases super-exponentially (Felsenstein 2004). This makes it impractical to apply  
390 quartet-based methods to trees with many taxa.

391 A solution to these problems is to estimate gene tree topologies directly, as many different  
392 methods can be used to accurately infer the topology at a locus. Once gene trees have been  
393 reconstructed from a large number of loci, the counts of discordant topologies can be used in  
394 much the same way as ABBA and BABA sites are in the  $D$  test. In fact, Huson et al. (2005)  
395 proposed such a test, using a statistic they called  $\Delta$ . Significance in genome-scale datasets can be  
396 evaluated by bootstrap-sampling the estimated gene trees (Vanderpool et al. 2020) or by  
397 assuming a  $\chi^2$  distribution (Suvorov et al. 2021), with  $\Delta = 0$  again representing the null  
398 hypothesis under ILS alone. While  $\Delta$  has greater potential to be affected by sources of technical  
399 error such as systematic bias in gene tree inference—and may have limited power to detect very  
400 ancient introgression—it has the advantage of being more robust to the infinite-sites assumption  
401 and allows for testing of introgression along deep, internal branches of a phylogeny. Therefore,  $\Delta$

402 represents a straightforward way to test for introgression using a small number of additional  
403 assumptions.

404 Estimated gene trees can also be used as input to phylogenetic network methods. These methods  
405 construct a likelihood or pseudolikelihood function that is explicitly derived from a phylogenetic  
406 network model, for which parameters can then be estimated using either maximum-likelihood or  
407 Bayesian approaches. Phylogenetic network methods can handle several different data types  
408 (which will be discussed in subsequent sections), but some of them can make inferences using  
409 only gene tree topologies as input. *PhyloNet* (Than et al. 2008, Wen et al. 2018) infers networks  
410 directly from gene tree topologies using either maximum likelihood or maximum pseudo-  
411 likelihood. Similarly, *SNaQ* (Solis-Lemus & Ane 2016) estimates a network with reticulation  
412 edges via maximum pseudo-likelihood using quartet concordance factors (Baum 2007)—  
413 essentially just the counts of the three possible unrooted tree topologies. The principles outlined  
414 in previous sections apply equally well to these methods, showing how phylogenetic network  
415 approaches can detect, polarize, and estimate the rate of introgression events in a tree with a  
416 minimum of five taxa. Additionally, they can be applied to species trees with many more than  
417 five taxa, making use of all available information available. We will discuss phylogenetic  
418 network methods in more detail later, in the section entitled “*Application and interpretation of*  
419 *methods for inferring introgression*”.

## 420 **Detecting introgression using coalescence times**

421 While much can be learned about introgression from the frequency of gene tree topologies alone,  
422 including additional information about the distribution of coalescence times can lead to much  
423 richer inferences. Some advantages of including coalescence times include more flexibility in  
424 inferring introgression between non-sister species, detection of introgression between sister taxa,  
425 and distinguishing introgression from ancestral population structure. In the following sections we  
426 expand on the expected effects of introgression on coalescence times and branch lengths,  
427 followed by a description of how this information is used in concert with gene tree frequencies to  
428 make inferences about introgression.

## 429 ***Detecting introgression using signals of pairwise divergence***

430 Just as was the case for gene tree topologies, it is possible to make inferences about introgression  
431 by studying violations of expected patterns of pairwise coalescence times under an ILS-only  
432 model. As previously mentioned, one of these expected patterns is a symmetry in coalescence  
433 times between the two pairs of non-sister taxa in a quartet (Figure 1, bottom). If one pair of non-  
434 sister taxa has more recent coalescence times on average than the other, post-speciation  
435 introgression between that pair is a likely explanation. Coalescence times can be approximated  
436 using simple measures of pairwise sequence divergence, assuming an infinite sites model (or at  
437 least that genetic distance is proportional to coalescence time). Therefore, one of the simplest  
438 ways to test for introgression is to test for an asymmetry in pairwise sequence divergence. This  
439 logic has been informally applied to test for introgression (Brandvain et al. 2014) and has  
440 recently been formalized in several test statistics including  $D_3$  (Hahn and Hibbins 2019) and the

441 branch-length test (Suvorov et al 2021).  $D_3$  is straightforward, and has the following definition  
442 (changed from the original to be consistent with the notation used here):

$$443 \quad D_3 = \frac{d_{P_2P_3} - d_{P_1P_3}}{d_{P_2P_3} + d_{P_1P_3}}$$

444 Where  $d$  denotes the genetic distance between the specified populations. This statistic takes the  
445 same general form as the  $D$ -statistic, where the relevant difference in the numerator is  
446 normalized by the sum of the two values in the denominator. Like the  $D$ -statistic, significance of  
447  $D_3$  can be evaluated using a block-bootstrap. A major advantage of  $D_3$  over site-pattern based  
448 tests is that it does not require data from an outgroup—it only needs one sample from three  
449 ingroup species. As with  $D$ ,  $D_3$  can only detect introgression between non-sister lineages.

#### 450 ***Characterizing introgression using reconstructed gene trees with branch lengths***

451 Using pairwise divergences between only non-sister taxa ignores information about the full  
452 distribution of coalescence times within different gene tree topologies. More information is  
453 contained within these branch lengths, allowing for estimation of the timing and direction of  
454 introgression in a quartet. Because introgressing taxa can coalesce via either introgression  
455 (Figure 4A, blue) or speciation (Figure 4A, red) depending on the history at a locus, a bimodal  
456 distribution arises when coalescence times are measured across loci (Figure 4A). This  
457 distribution is not expected under ILS alone, and can therefore be used to test for introgression.  
458 In addition, the more recent peak provides information about the timing of introgression, while  
459 the frequency of gene trees under this peak compared to the older peak provides information on  
460 the rate of introgression.

461 This approach to characterizing introgression is implemented in the software *QuIBL*  
462 (Quantifying Introgression via Branch Lengths) (Edelman et al. 2019). *QuIBL* takes gene trees  
463 with inferred branch lengths as input, using maximum-likelihood to infer whether one  
464 distribution (ILS-only) or two distributions (ILS + introgression) is a better fit. If two  
465 distributions is a better fit, then introgression between non-sister species is inferred. *QuIBL* may  
466 also be able to infer the timing and rate of introgression using information contained within these  
467 distributions.

468 The direction of introgression uniquely affects the coalescence times of the non-sister pair of  
469 species uninvolved in introgression (Figure 2C, Figure 4B). For example, the direction of  
470 introgression between  $P_2$  and  $P_3$  has predictable effects on the coalescence time between  $P_1$  and  
471  $P_3$ . When introgression occurs from  $P_3$  into  $P_2$  (Figure 4B, left),  $P_2$  traces its ancestry through  
472 the  $P_3$  lineage at introgressed loci (note that while the direction of introgression is typically  
473 described forward in time, the coalescent process occurs backwards in time). Because of this,  
474 divergence between  $P_1$  and  $P_3$  is unchanged by introgression in this direction. By contrast, when  
475 introgression is from  $P_2$  into  $P_3$  (Figure 4B, right),  $P_3$  traces its ancestry through the  $P_2$  lineage  
476 at introgressed loci. This allows  $P_3$  to coalesce with  $P_1$  earlier than it normally would, which  
477 decreases the divergence between  $P_1$  and  $P_3$ .

478 These genealogical processes lead to general predictions that can be used to infer the primary  
479 direction of introgression between taxa. Gene trees that are concordant with the species tree can  
480 be used as a baseline for the expected amount of  $P1$ - $P3$  divergence; although these trees can  
481 arise from ILS at introgressed loci, the effect of the direction will not be manifest since they are  
482 concordant. By comparing this baseline divergence to the amount of  $P1$ - $P3$  divergence in gene  
483 trees consistent with a history of introgression, the direction of introgression can be inferred.  
484 Lower  $P1$ - $P3$  divergence in the latter class of trees provides evidence for  $P2 \rightarrow P3$  introgression,  
485 but does not necessarily rule out the other direction (i.e. there could simply be less gene flow in  
486 the other direction). Alternatively, if  $P1$ - $P3$  divergence is the same in both topologies, then  
487 introgression is primarily  $P3 \rightarrow P2$ . This logic to polarizing introgression is used by the  $D_2$   
488 statistic (Hibbins and Hahn 2019) and the *DIP* method (Forsythe et al. 2020).

489 Finally, *PhyloNet* (Than et al. 2008, Wen et al. 2018) is able to infer phylogenetic networks with  
490 reticulation edges (i.e. discrete introgression events) from gene trees with branch lengths using  
491 maximum likelihood. Based on the previously discussed patterns, this method should be capable  
492 of accurately estimating the presence, timing, direction, and rate of introgression by making use  
493 of all available information on the distribution of coalescence times. It can also estimate multiple  
494 independent events on the same species tree, and on trees with more than five taxa. As we  
495 discuss in a following section, it is also capable of detecting the signals of introgression between  
496 sister species.

#### 497 ***Distinguishing introgression from ancestral population structure***

498 In addition to being generated by introgression, asymmetric gene tree topology frequencies can  
499 arise from certain kinds of ancestral population structure (Slatkin and Pollack 2008, Durand et al.  
500 2011, Lohse and Frantz 2014). The scenario that generates asymmetries imagines that the  
501 population ancestral to all three species is split into at least two subpopulations, such that the  
502 ancestors of  $P3$  are more closely related to either the ancestors of  $P1$  or  $P2$  (but not both)  
503 (Supplementary Figure 1A). Because the gene tree topologies in this ancestral species will be  
504 skewed toward relationships joining  $P3$  and one of the sister lineages, this scenario can lead to a  
505 significant asymmetry in gene tree topologies even in the absence of post-speciation  
506 introgression (Durand et al. 2011). This will also result in a slight asymmetry of genome-wide  
507 pairwise divergence times, since the more common discordant tree will contribute more to the  
508 average value. All of this means that ancestral structure can result in false positives when testing  
509 for introgression using simple patterns of asymmetry.

510 Fortunately, while these two scenarios are indistinguishable using only gene tree topologies  
511 alone, they are distinguishable when using the distribution of branch lengths. Under ancestral  
512 population structure, divergence between the sister taxa in whichever discordant gene tree  
513 becomes more frequent will be higher than it would be under introgression. Lohse and Frantz  
514 (2014) incorporated the expected branch length differences in these two models into a  
515 maximum-likelihood framework, which was then used to confirm the signal of human-  
516 Neanderthal introgression that was originally uncovered by the  $D$ -statistic. Additionally,  
517 ancestral population structure is not expected to result in a bimodal distribution of coalescence

518 times. This means that methods capable of detecting two peaks of coalescence, such as *QuIBL*  
519 and *PhyloNet*, should also be robust to the effects of population structure.

### 520 ***Detecting introgression between sister species***

521 Introgression between sister species is very difficult to detect using a single sample from each  
522 species. The classic asymmetry patterns described in previous sections do not apply in this  
523 scenario, either for gene tree topologies or coalescence times. While introgression between sister  
524 species should lead to an increased variance in coalescence times compared to an ILS-only  
525 model, this signal is easily confounded by other processes such as non-equilibrium demography  
526 or linked selection (Cruickshank and Hahn 2014; Roux et al. 2016; Sethuraman et al. 2019).  
527 These limitations have typically been addressed by combining two alternative sources of  
528 information: 1) polymorphism data for the two introgressing species, and 2) local reductions in  
529 between-species divergence relative to a genome-wide baseline.

530 Most available methods for inferring introgression between sister taxa are not phylogenomic in  
531 multiple senses: they typically require polymorphism data, they often identify locally  
532 introgressed regions rather than genome-wide signals, and they do not explicitly test against an  
533 ILS-only case. Genome scans using summary statistics such as  $F_{ST}$  (Wright 1949) and  $d_{xy}$  (Nei  
534 and Li 1979) are common, though relative measures of divergence such as  $F_{ST}$  are confounded  
535 by natural selection when used for this task (Charlesworth 1998, Noor and Bennett 2009,  
536 Nachman and Payseur 2012, Cruickshank and Hahn 2014). There are multiple statistics based on  
537 minimum pairwise distances between multiple haplotypes in two species that avoid problems  
538 caused by selection (Joly et al. 2009, Geneva et al. 2015, Rosenzweig et al. 2016), and new  
539 machine learning methods combine multiple summary statistics into a single comparative  
540 framework that is powerful and robust (e.g. Schrider et al. 2018). However, these methods also  
541 usually require coalescent simulation under known demographic history to evaluate patterns of  
542 introgression, and this information is not always available.

543 None of the aforementioned limitations mean that genome-wide tests with one sample per  
544 species are not possible. Introgression between sister taxa—at least when it occurs in relatively  
545 discrete pulses—should result in the same multimodal distribution of coalescence times  
546 described above for non-sister taxa. This may be the most promising avenue for a genome-wide  
547 test of sister introgression when only one sample per species is available, since coalescence times  
548 for two species should follow an exponential distribution under ILS alone. Nevertheless, no  
549 methods have been developed to date that explicitly test for this pattern (*QuIBL* can only infer it  
550 for non-sister taxa). However, *PhyloNet* appears to be capable of reliably inferring introgression  
551 (including estimating the timing and rate) between sister taxa using gene trees with branch  
552 lengths using this signal (Wen and Nakhleh 2018), at least when nested within a tree containing  
553 more taxa. Despite this, the direction of introgression between sister taxa may not be inferable  
554 from only one sample per species.

555 Finally, while introgression between extant sister species is not detectable using gene tree  
556 frequencies, this may not necessarily be the case for introgression between ancestral sister  
557 lineages. Several studies have now shown that when introgression occurs between *P3* and the

558 ancestor of  $P1$  and  $P2$  (event 2 in Figure 2), it becomes possible under specific conditions for  
559 both discordant gene tree topologies to become more common than the species tree topology,  
560 while remaining at equal frequencies (Solís-Lemus et al. 2016, Long and Kubatko 2018, Jiao and  
561 Yang 2020). It should be possible in principle to infer introgression using this pattern, but it  
562 requires sufficiently high rates of introgression to result in the anomalous trees, in addition to  
563 independent knowledge of the species tree topology.

## 564 **Application and interpretation of methods for inferring introgression**

### 565 *Evaluating the power to detect and characterize introgression*

566 To illuminate many of the patterns and approaches discussed in this review, we conducted a  
567 small simulation study using *ms* (Hudson 2002) and *Seq-Gen* (Rambaut and Grassly 1997). We  
568 used the five introgression scenarios shown in Figure 2, as well as one scenario with only ILS  
569 and several additional scenarios involving ghost introgression (Supplementary Figure 2). For  
570 each set of conditions, we performed 100 replicate simulations each consisting of 3000 gene  
571 trees with branch lengths. We simulated 1kb per locus using *Seq-Gen* with  $\theta = 0.005$  per  $2N$   
572 generations. We evaluated the performance of three different test statistics designed to capture  
573 slightly different information about introgression:  $D$ ,  $D_3$ , and  $\Delta$ . In addition, we applied the  
574 InferNetwork\_ML method (Yu et al. 2014) in *PhyloNet*, which infers a phylogenetic network  
575 using maximum-likelihood. For the three test statistics, we evaluated significance by bootstrap-  
576 resampling the gene trees in each dataset to estimate the sampling variance. The z-score obtained  
577 from bootstrap-resampling was used to estimate a two-tailed  $p$ -value. The method we use in  
578 *PhyloNet* evaluates the fit of a phylogenetic network internally (Yu et al. 2012) using a  
579 combination of the model selection measures AIC (Akaike 1974), AICc (Burnham and Anderson  
580 2002), and BIC (Schwarz 1978). For our purposes, a positive result was taken as any result  
581 where *PhyloNet* selected a network over a strictly bifurcating tree. See Supplementary Table 1  
582 for the simulation parameters used for each condition.

583 The power of each method to detect introgression under each scenario is shown in Figure 6. All  
584 four methods yielded low false positive rates in the presence of high ILS but no introgression,  
585 confirming that they are effective tests against an ILS-only null hypothesis. For non-sister taxa,  
586 *PhyloNet* was always capable of identifying introgression, while the power of the other methods  
587 was strongly affected by the direction of introgression. A reduction of power for  $P1 \rightarrow P3$   
588 introgression is consistent with the effect of direction on gene tree branch lengths described  
589 above, but the magnitude of the reduction is somewhat surprising: there is almost three times as  
590 much power to detect  $P3 \rightarrow P1$  introgression. Of the four methods, only *PhyloNet* appears  
591 capable of reliably inferring introgression between sister lineages, again consistent with  
592 expectations.

593 The  $D$  and  $\Delta$  statistics, as well as *PhyloNet*, did not give significant results when introgression  
594 occurred between  $P1$  and an unsampled ingroup lineage. The  $D_3$  statistic, interestingly, does  
595 appear to be sensitive to this scenario, at least when the ghost population is the donor. This  
596 suggests that patterns of pairwise divergence may be especially useful for detecting introgression  
597 with unsampled populations. When introgression occurs between  $P1$  and an outgroup ghost



598 lineage, there is no effect when the ghost is the recipient, while all four methods are strongly  
599 affected when the ghost is the donor. These observations are consistent with expectations for  
600 ghost populations, highlighting the importance of careful interpretation of the potential taxa  
601 involved in a positive result. In this case, all methods appear to suggest introgression between  $P2$   
602 and  $P3$ , even though neither of these lineages was involved in the introgression. This occurs  
603 because introgression from outside the rooted triple draws  $P1$  to the outside as well, leaving  $P3$   
604 more closely related to  $P2$ .

605 In addition to testing for the presence of introgression, we evaluated the ability of *PhyloNet* to  
606 infer the direction of introgression, and of several methods to infer the rate of introgression. We  
607 evaluated the ability of *PhyloNet* to correctly identify the taxa involved, the donor and recipient  
608 lineages, and the rate of introgression. For the two conditions involving introgression between  
609 non-sister taxa, we additionally estimated the rate of introgression using the  $D_p$  statistic and an  
610 analogous version of the  $\Delta$  statistic where the count of the concordant tree topology was added to  
611 the denominator; we refer to this statistic as  $\Delta_p$ .

612 We found that *PhyloNet* was highly accurate at identifying the taxa and direction for  $P1 \rightarrow P3$   
613 introgression (Supplementary Figure 3). However, somewhat surprisingly, it often failed to  
614 identify the taxa involved when introgression was  $P3 \rightarrow P1$  (although it always correctly  
615 identified that introgression had occurred somewhere). While it is more difficult to detect  
616 introgression in the  $P1 \rightarrow P3$  direction, once it is detected it appears that the additional signal in  
617 gene tree branch lengths makes it easier for *PhyloNet* to infer the direction. For sister lineages,  
618 *PhyloNet* always correctly identified the taxa, but cannot accurately infer the direction. However,  
619 *PhyloNet* must always specify the direction of introgression (see below for more explanation),  
620 and its behavior differs between scenarios. For introgression between extant sister species, the  
621 direction of introgression appears to be assigned randomly, while for ancestral sister species  
622 introgression is always inferred to be in one direction. For the rate of introgression, *PhyloNet*  
623 appears to slightly overestimate the true rate under all scenarios in which it correctly identified  
624 introgression (Supplementary Figure 4). By contrast,  $D_p$  and  $\Delta_p$  tend to slightly underestimate the  
625 rate of introgression between non-sister taxa (Supplementary Figure 4).

### 626 ***Inferring the number of introgression events***

627 A major challenge that remains in the inference of introgression is how to assess the fit of  
628 different numbers of introgression events inferred on the same tree. The mostly widely used  
629 methods are formulated to test for the presence of introgression versus no introgression, but  
630 provide no rigorous way to evaluate the number of distinct introgression events. One approach is  
631 to perform many quartet-based tests, and then to infer the most parsimonious set of introgression  
632 events by collapsing sets of positive tests that share the same ancestral populations (Pease et al.  
633 2016, Suvorov et al. 2021). However, this approach is highly conservative, as it can collapse  
634 cases where there truly are multiple instances of post-speciation introgression within a clade.  
635 Additionally, it requires large datasets and the piecing together of many quartets, which makes it  
636 impractical in many cases. Nonetheless, it can be used to generate a conservative estimate for the  
637 minimum number of introgression events.

638 Even with likelihood methods, estimating the number of introgression events is not a solved  
639 problem. One issue is that adding additional parameters to the likelihood model always improves  
640 the likelihood score. This makes it necessary to penalize model complexity when comparing  
641 estimated likelihoods. Unfortunately, the information measures that are classically used to  
642 perform model selection, such as AIC and BIC, do not adequately scale with the increased  
643 complexity of adding a new reticulation to a phylogenetic network. This is because adding a new  
644 reticulation does not just add a new model parameter—it adds a whole new space of possible  
645 networks, with different taxa involved in introgression, at different times and in different  
646 directions (Blair and Ané 2020). AIC and BIC penalize the increased complexity of model  
647 parameters, but not the increased complexity of models within a set of parameters. The problem  
648 is greater for methods based on pseudo-likelihood such as *SNaQ*, because these information  
649 measures are not intended for pseudo-likelihood estimates. Bayesian approaches such as those  
650 implemented in *PhyloNet* (Wen and Nakhleh 2018) and *SpeciesNetwork* can incorporate  
651 appropriate penalties for model complexity, but unfortunately scale poorly to larger datasets and  
652 larger numbers of reticulations (Elworth et al. 2019).

653 While no methods currently exist that can both explicitly penalize model complexity and scale to  
654 large datasets, there are several alternate approaches available for assessing the fit of  
655 phylogenetic networks. One simple, empirical approach is to use a slope heuristic where  
656 networks are inferred across different numbers of reticulations, and the best network is taken as  
657 the least complex one after which the likelihood score appears to stop improving. This is the  
658 method recommended for use with *SNaQ* (Solís-Lemus and Ané 2016). *PhyloNet* has methods  
659 that can evaluate the fit of a network using  $k$ -fold cross-validation or parametric bootstrapping  
660 (Yu et al. 2014), which can both address this problem. Finally, a promising approach from Cai  
661 and Ané (2020) involves using the multispecies network coalescent to calculate the quartet  
662 concordance factors expected from an estimated network. A goodness-of-fit function is then used  
663 to evaluate the fit of these expected concordance factors to those observed in the data. This is  
664 similar to the method implemented in *admixturegraph* (Leppälä et al. 2017) for use with  $D$   
665 statistics (see next section).

### 666 ***Visualizing and interpreting phylogenetic networks***

667 When visualizing inferred phylogenetic networks, reticulations represent the histories of loci that  
668 have introgressed. Visually, the relative placement, orientation, and length of these reticulations  
669 imply specific information about the timing and direction of introgression, as well as the identity  
670 of the species involved. However, not all phylogenetic networks are constructed from the same  
671 underlying models, and therefore they may not always convey the same information (Huson and  
672 Bryant, 2005). As a result, choices for network visualization that are primarily stylistic can  
673 unintentionally imply specific introgression processes. In this section we discuss these different  
674 visualizations and how to interpret them.

675 One important distinction when visualizing networks is the contrast between introgression that  
676 occurs among extant lineages and introgression that results in the formation of a new lineage.  
677 Supplementary Figure 5A depicts the former scenario, which corresponds to the introgression  
678 scenarios considered in this paper thus far. In such cases, a single horizontal reticulation is

679 typically used to connect the two taxa involved. Such visualizations do not naturally convey any  
680 information about the direction of introgression. By contrast, methods that assume the formation  
681 of an admixed population (e.g., Bertorelle and Excoffier 1998, Wang 2003) or hybrid species  
682 (e.g., Meng and Kubatko 2009) often use the visualization shown in Supplementary Figure 5B,  
683 where reticulations connect each parent lineage to the newly formed lineage. This representation  
684 implies a directionality of introgression: from the two parent lineages into the newly formed  
685 lineage. In both cases, a horizontal reticulation edge is used to denote the instantaneous exchange  
686 of alleles between the involved lineages. Supplementary Figure 5C shows an example using non-  
687 horizontal branches, which may imply a period of branching off and independent evolution from  
688 the parent species before the hybrid lineage is formed (e.g., Patterson et al 2012, Yu *et al.* 2014,  
689 Zhang *et al.* 2018). Alternatively, this could represent "standard" introgression involving a now-  
690 extinct species, in which case the extinct lineage was the donor in the introgression scenario. In  
691 all three cases, the placement of the reticulation edge conveys information about the timing of  
692 introgression and/or lineage formation.

693 The key take-away from this last representation is that non-horizontal reticulation edges often  
694 imply directionality, with the introgressed alleles travelling toward the tips. Unfortunately, many  
695 automated methods for visualizing species networks do not allow strictly horizontal edges—  
696 instead, all reticulations must have a bifurcating "parent" node that occurs closer to the root than  
697 the "daughter" node, which has two incoming lineages. This was the behavior observed in  
698 *PhyloNet* in the previous section. To highlight how different network visualizations can  
699 potentially be (mis-)interpreted, with particular emphasis on the direction of introgression, we  
700 plotted the same inferred networks using four popular tools (Figure 5): *Dendroscope* (Huson and  
701 Scornavacca 2012), *IcyTree* (Vaughan 2017), *PhyloPlots*, which is part of the Julia package  
702 *PhyloNetworks* (Solís-Lemus et al. 2017), and *admixturegraph* (Leppälä et al. 2017). The  
703 networks were inferred using *PhyloNet* on simulated gene trees from the two non-sister  
704 introgression scenarios (i.e. both  $P1 \rightarrow P3$  and  $P3 \rightarrow P1$ ) discussed in the previous section. For  
705 *admixturegraph*, we simply plotted the outcome of applying the *D*-statistic to the data under both  
706 scenarios.

707 In *Dendroscope*'s visualizations (Figure 5A, 5B), the position of the hybrid node (the node with  
708 two parents) is made clear, but it is not clear which parent corresponds to a history of  
709 introgression vs. the species tree history, since both are represented using a curved blue line (and  
710 therefore both resemble reticulation edges). As a result, the direction of introgression confounds  
711 accurate representation of the underlying introgression scenario. For the  $P3 \rightarrow P1$  direction  
712 (Figure 5A), the visualization strongly implies that *P1* is a hybrid species that formed from  
713 hybridization between *P2* and *P3*. While this representation accurately conveys the fact that *P1*  
714 is the recipient of introgressed alleles, it unfortunately suggests that *P2* was involved in  
715 hybridization when it was not. The  $P1 \rightarrow P3$  visualization (Figure 5B) is easier to interpret,  
716 because one of the blue edges cannot possibly represent an introgression history. Additionally,  
717 the curvature of the blue edges suggests a non-horizontal reticulation, which may imply a period  
718 of independent evolution. However, in this case it is purely stylistic as the network does not  
719 contain any branch lengths. Finally, it is important to note that all the visualizations we discuss  
720 do not take branch lengths into account, so the placement of the reticulation edges within

721 branches of the species tree are arbitrary and do not convey information about the timing of  
722 introgression. *IcyTree* and *PhyloPlots* are capable of plotting networks with branch lengths, in  
723 which case the timing of introgression within lineages can be displayed. Since our primary  
724 concern is with the direction of introgression, we have not shown these visualizations.

725 *IcyTree* uses a different style of visualization (Figure 5C, D). A dashed line represents the  
726 reticulation edge, which branches off from the donor population and enters the recipient  
727 population. This allows the introgression and species tree histories to be more visually distinct,  
728 while still depicting the direction of introgression. However, it implies that a lineage branched  
729 off from the donor and underwent a period of independent evolution before entering the  
730 recipient, which did not happen in either case. As the network is plotted without branch lengths,  
731 the point at which the reticulation leaves the donor branch is arbitrary. *PhyloPlots* (Figure 5E, F)  
732 visually distinguishes the reticulation edge (light blue) from the species tree history (black) while  
733 explicitly labelling the hybrid node. The reticulation edge is not horizontal, erroneously implying  
734 some period of independent evolution, though it does effectively convey the direction of  
735 introgression. The distinct coloration of each history, in combination with labelling of the hybrid  
736 node, means that the direction of introgression can be easily visualized. Finally, *admixturegraph*  
737 (Figure 5G, 5H) plots the network solely from the results of a series of *D* tests. This means that  
738 no inference of directionality is possible. As with *Dendroscope*, this method plots phylogenetic  
739 networks as admixture graphs, which have the same issues with implied directionality and hybrid  
740 speciation. In our case, this results in *PI* being the implied recipient of introgression regardless  
741 of the true direction.

742 The message we hope to convey from this discussion is that it is very difficult to simultaneously  
743 visualize the direction of introgression and to preserve the underlying model of hybridization.  
744 This is especially challenging for cases when network visualization needs to be automated,  
745 because the standard computational representation of phylogenetic networks, the Extended  
746 Newick format (Cardona et al. 2008), requires labeling of parent and daughter nodes, and  
747 therefore implies directionality any time a hybrid node is inferred. “Tube tree” representations  
748 like the ones we use for figures in this paper (e.g. Figure 4) can be effective for individual cases,  
749 but to our knowledge no automated approaches exist as of yet that can accurately convey all the  
750 necessary information. In general, care should be taken not to over-interpret phylogenetic  
751 network visualizations.

## 752 **Conclusions**

753 In conclusion, several methodological and technical challenges remain in the inference of  
754 introgression, including: more accurate estimation of the rate, timing, and direction of  
755 introgression; detection of introgression between sister taxa; spurious results generated by  
756 unsampled lineages; inference of the number of introgression events in a clade; and accurate  
757 automated visualization of phylogenetic networks. Despite these challenges, currently available  
758 approaches have remarkable power to detect and characterize introgression under a wide variety  
759 of conditions, especially when used in a complementary fashion. Overall, these methods will  
760 continue to reveal the nature and influence of introgression throughout the natural world.

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762 We thank Leonie Moyle, Rafael Guerrero, Claudia Solís-Lemus, Cécile Ané, Mia Miyagi, and  
763 Luay Nakhleh for helpful comments and discussion. This work was supported by National  
764 Science Foundation grant DEB-1936187.

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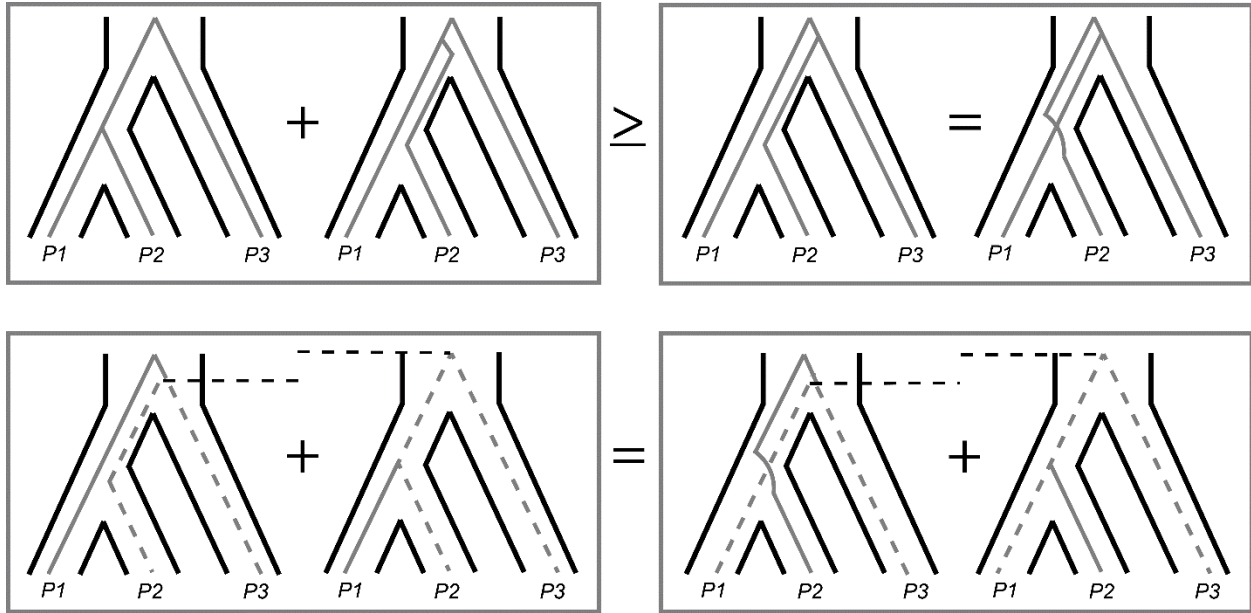
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781 *Figure 1:* Expected gene tree topologies and coalescence times under ILS only. For a rooted  
 782 triplet, four topologies are possible (top row): two concordant with the species tree, which can  
 783 result either from lineage sorting or ILS (top left), and two that are discordant with the species  
 784 tree and arise from ILS only (top right). The two concordant trees must be at least as frequent as  
 785 the two discordant trees, which are equally frequent to each other. For non-sister pairs of taxa—  
 786 either *P2-P3* (bottom left) or *P1-P3* (bottom right)—coalescence is expected to occur at one of  
 787 two times, depending on whether they coalesce first or second in a gene tree (grey dotted lines).  
 788 These expected times are symmetrical across gene trees, and so pairwise divergences between  
 789 the non-sister lineages are expected to be equal when averaged across loci.

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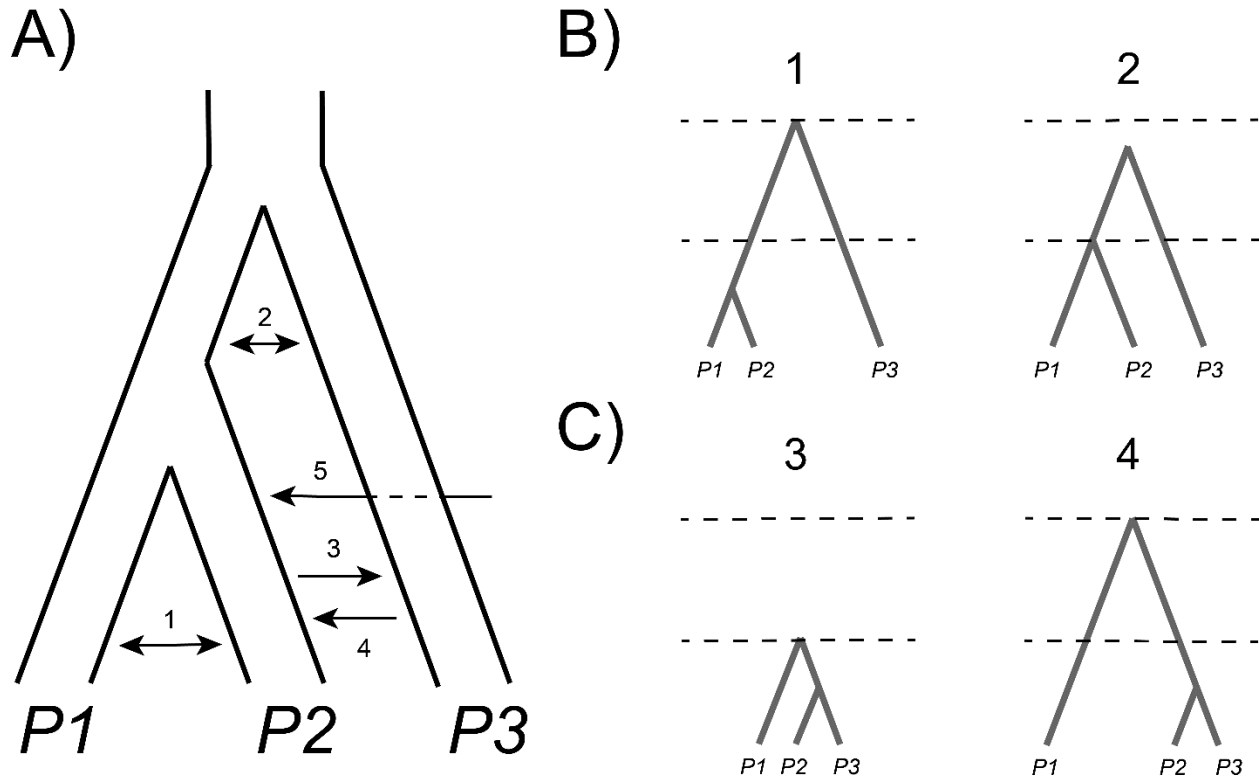
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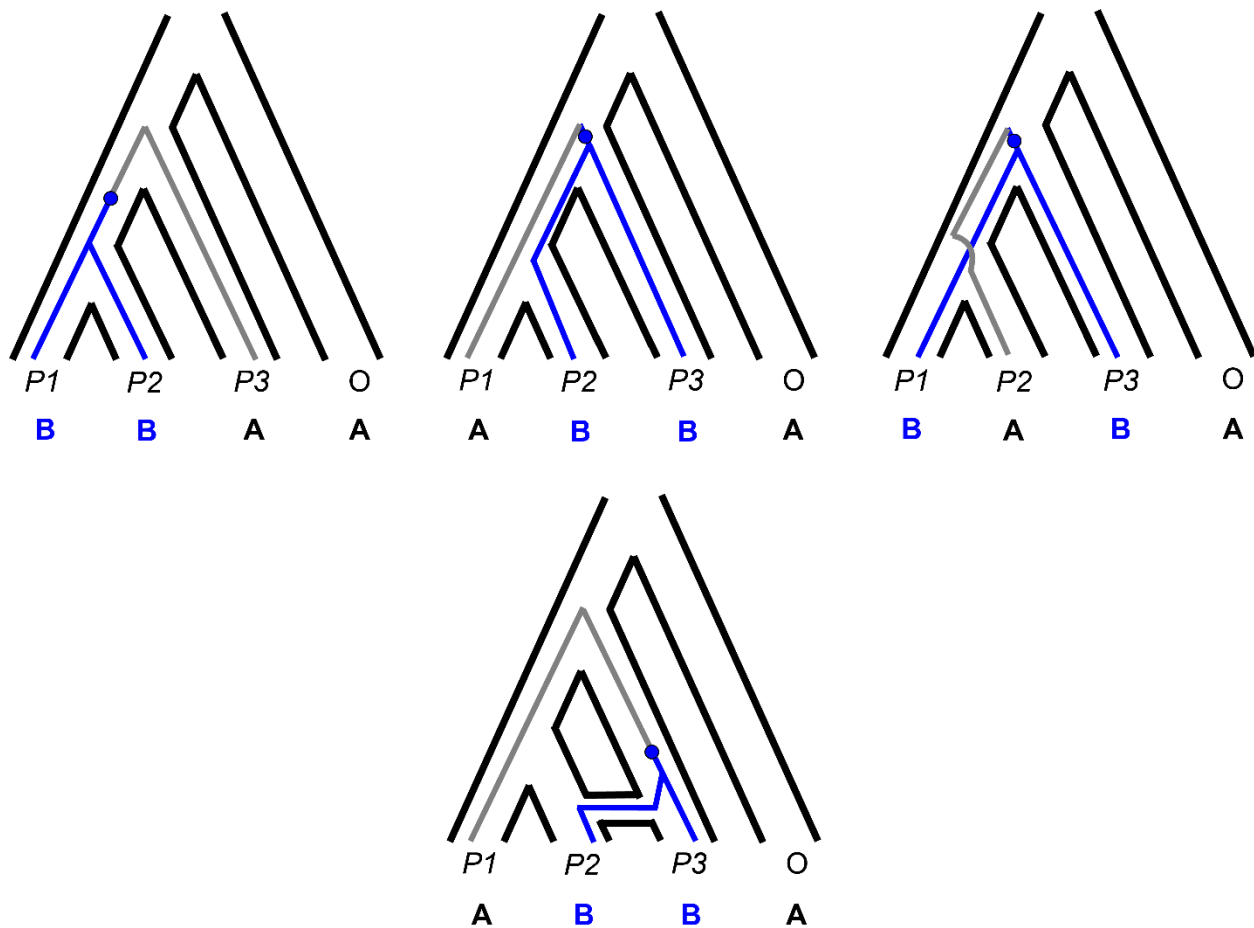
803 *Figure 2: An overview of detectable introgression scenarios for a rooted triplet, and their effects*  
 804 *on gene tree frequencies and branch lengths. A. The species tree relating three lineages.*  
 805 *Introgression can occur between extant (1) or ancestral (2) sister lineages, or between non-sister*  
 806 *taxa, with  $P3$  as either the recipient (3) or the donor (4). One of the sampled taxa may also be the*  
 807 *recipient of introgression from an unsampled taxon (5). B. Gene trees for introgression between*  
 808 *sister lineages. Introgression between sister taxa reduces divergence between the involved taxa*  
 809 *but does not generate discordant gene trees (events 1 and 2). In both trees the expected time to*  
 810 *coalescence for pairs of lineages in the absence of introgression is denoted with dashed*  
 811 *horizontal lines. C. Gene trees for introgression between non-sister lineages. When  $P3$  is the*  
 812 *recipient of introgression (event 3), discordant gene trees are generated uniting  $P2$  and  $P3$ . In*  
 813 *addition, divergence is reduced between both  $P2$  and  $P3$  and between  $P1$  and  $P3$ . When  $P3$  is the*  
 814 *donor of introgression (event 4) discordant gene trees are again generated uniting  $P2$  and  $P3$ . In*  
 815 *this case divergence is reduced only between  $P2$  and  $P3$ , while divergence is increased between*  
 816  *$P1$  and  $P2$ . In both trees the expected time to coalescence for pairs of lineages in the absence of*  
 817 *introgression is denoted with dashed horizontal lines. No example gene tree is shown for*  
 818 *introgression from a ghost lineage outside the triplet. The expectation is that these*  
 819 *events will generate topologies with  $P2$  pulled outside the clade, sister to the two unintrogressed*  
 820 *lineages.*

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826 *Figure 3:* Biallelic site patterns are informative of underlying gene tree topologies. With the  
827 exception of low levels of homoplasy, such patterns can only arise from mutations (blue) on  
828 internal branches of the local genealogy. The occurrence of the incongruent site patterns  
829 “ABBA” (top middle) and “BABA” (top right) are therefore expected to reflect the frequency of  
830 discordant gene tree topologies. With introgression between a specific non-sister species pair,  
831 one incongruent pattern (bottom) can increase in frequency over the other due to the underlying  
832 asymmetry in gene tree frequencies.

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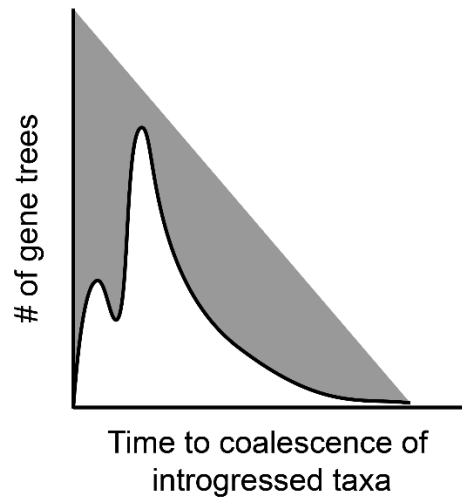
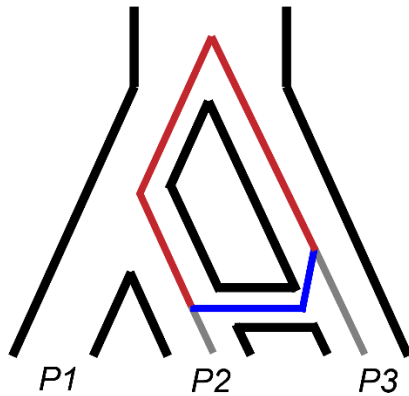
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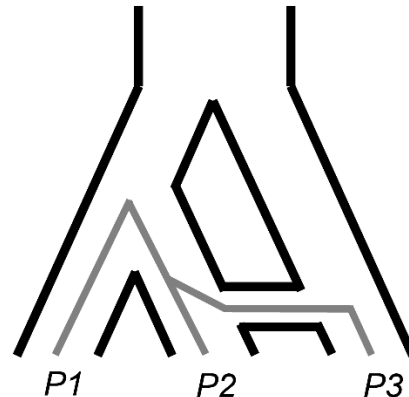
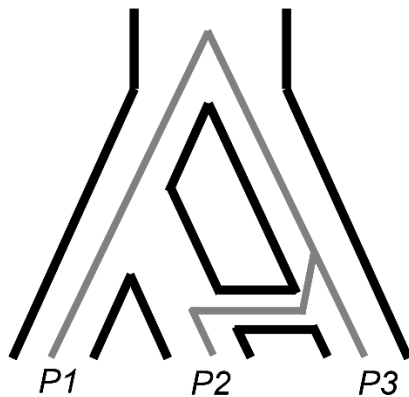
A)



B)

$P3 \rightarrow P2$

$P2 \rightarrow P3$



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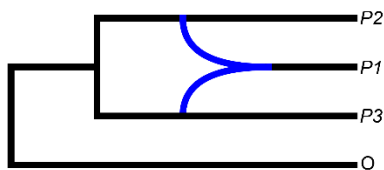
838 *Figure 4:* Coalescence times provide information on the timing, direction, and presence of  
 839 introgression. A) Post-speciation introgression between  $P2$  and  $P3$  allows them to coalesce more  
 840 quickly at introgressed loci (blue). This reduces their whole-genome divergence relative to  $P1$   
 841 and  $P3$ , an asymmetry that can be used to test for introgression. Since coalescence can now occur  
 842 at one of two times, after introgression (blue) or after speciation (red), it also results in a bimodal  
 843 distribution of coalescence times across loci (right figure). The more recent peak of this  
 844 distribution can be used to estimate the timing of introgression. B) The direction of introgression  
 845 between  $P2$  and  $P3$  affects the time to coalesce of  $P1$  and  $P3$  at introgressed loci.  $P2 \rightarrow P3$   
 846 introgression allows  $P1$  and  $P3$  to coalesce more quickly (right), reducing their divergence at  
 847 introgressed loci.

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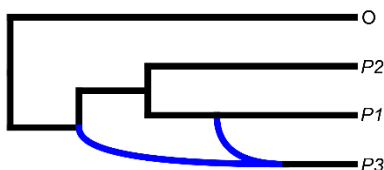
$P3 \rightarrow P1$

$P1 \rightarrow P3$

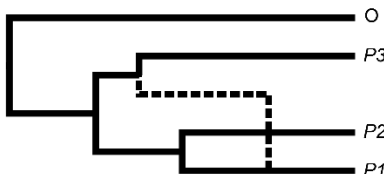
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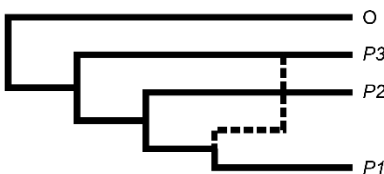
B)



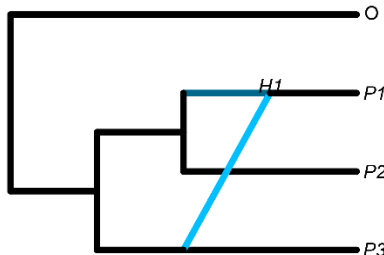
C)



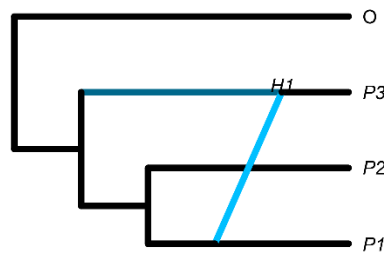
D)



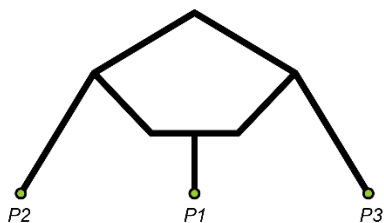
E)



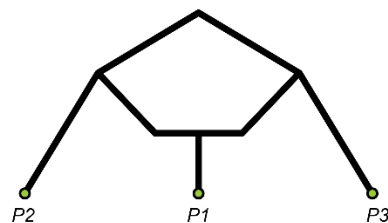
F)



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H)



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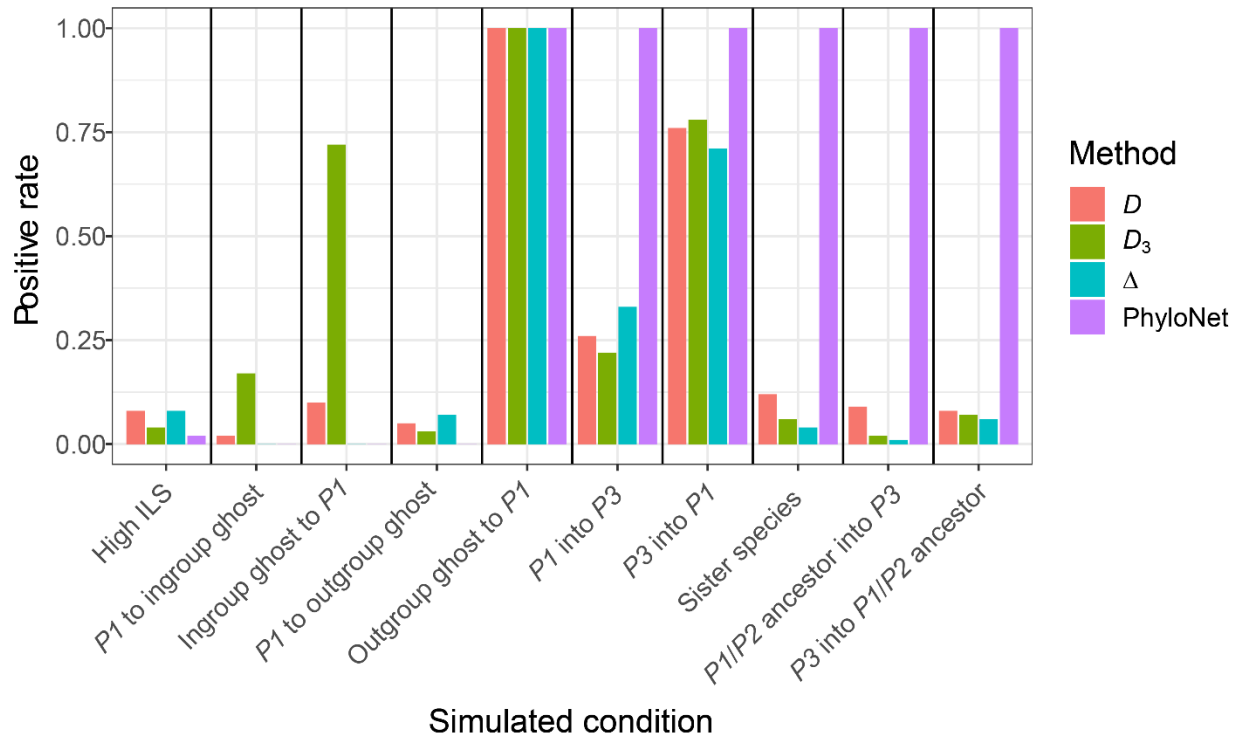
850 *Figure 5: Different visualizations of the same underlying phylogenetic networks. The left*  
851 *column comes from a network representing  $P3 \rightarrow P1$  introgression, while the right*  
852 *column comes from a network representing  $P1 \rightarrow P3$  introgression. The rows, from top to bottom, show*  
853 *visualizations from *Dendroscope* (A, B), *IcyTree* (C, D), *PhyloPlots* (E, F), and *admixturegraph**  
854 *(G, H), respectively.*

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860 *Figure 6: Power (y-axis) of four different methods (color legend) to infer the presence of*  
861 *introgression across ten different simulation conditions (x-axis). Power is measured as the*  
862 *proportion of tests that are significant; for the "High ILS" condition it therefore represents the*  
863 *false positive rate.*

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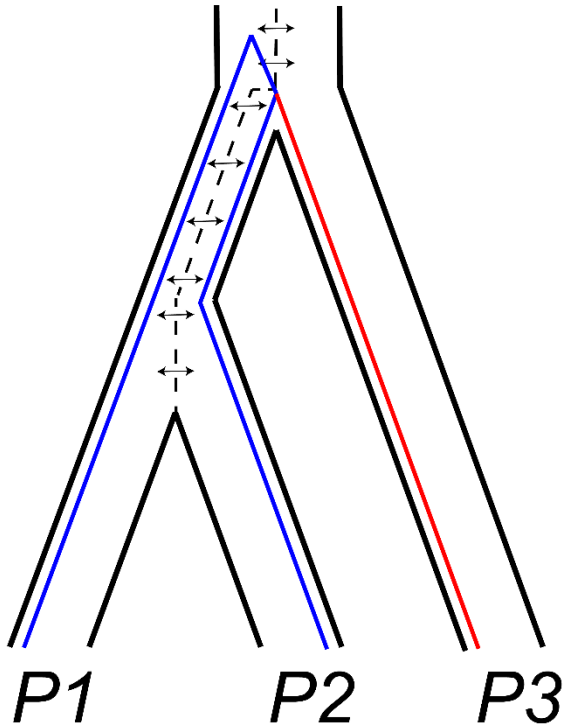
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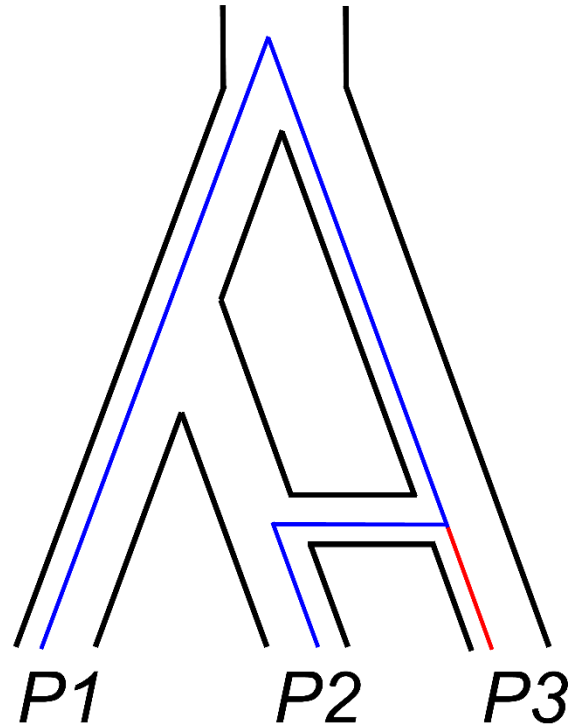
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A)



B)



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872 *Supplementary Figure 1: Distinguishing ancestral population structure (A) from introgression*  
 873 *(B). Persistent structure in the ancestral population of a quartet, which may or may not continue*  
 874 *after the first speciation event, can result in the same asymmetries in gene tree topologies and*  
 875 *divergence times that are expected from introgression between non-sister taxa. These two*  
 876 *scenarios are distinguishable by studying the distribution of branch lengths, in particular the*  
 877 *length of the tip branch leading to P3 (red).*

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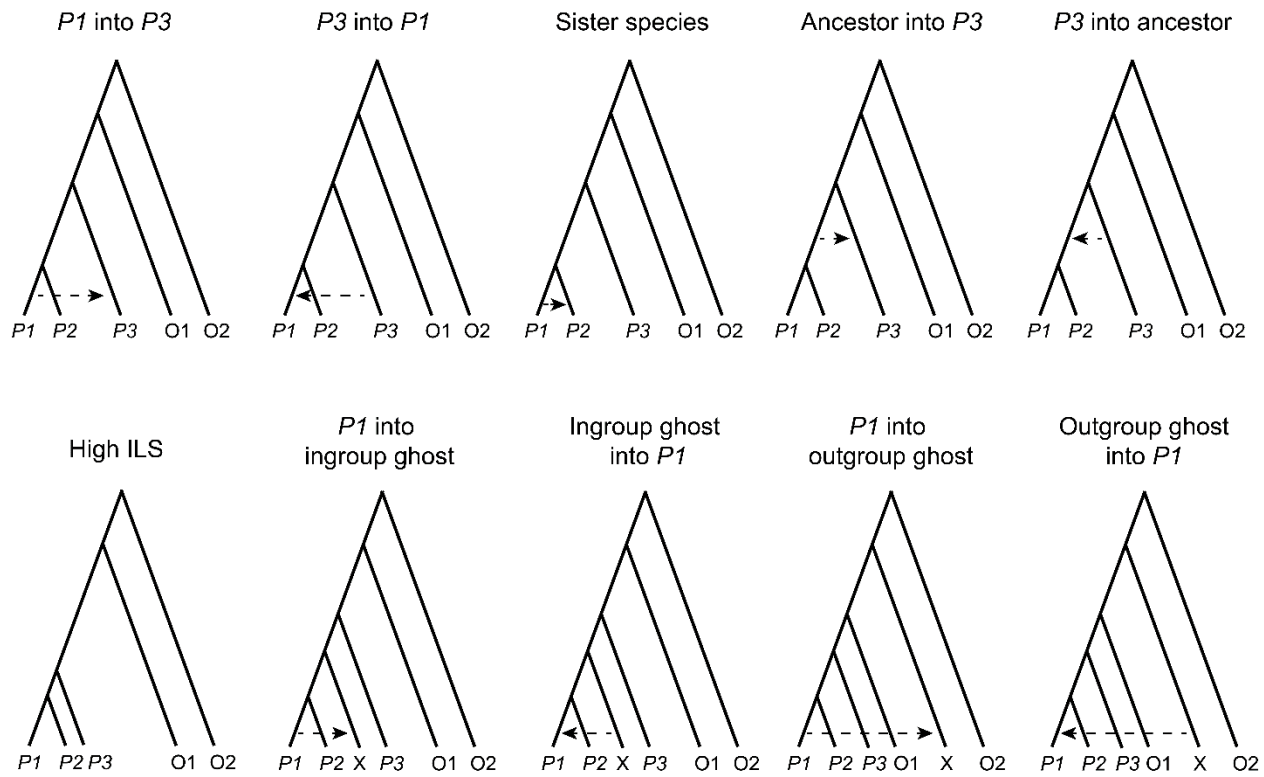
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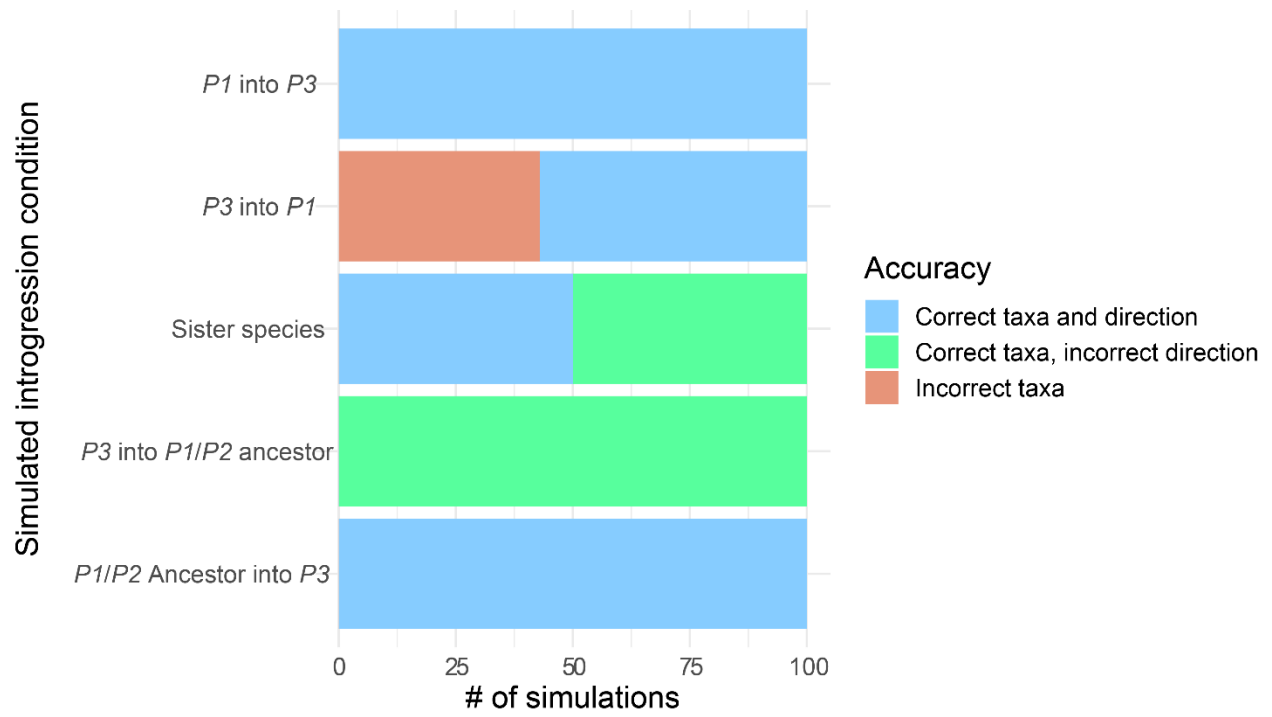
891 *Supplementary Figure 2: A visual overview of the ten different conditions used in our simulation*  
892 *study. Branch lengths are not to scale.*

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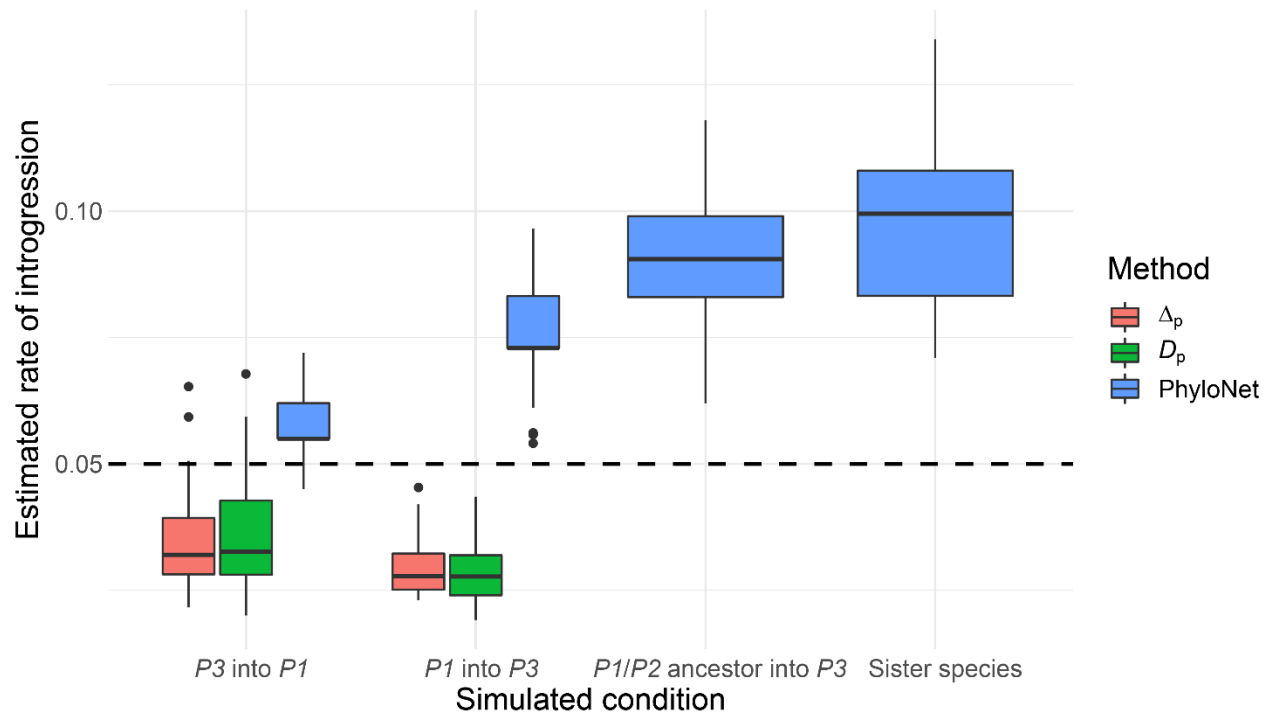
898 *Supplementary Figure 3: The power of PhyloNet to identify the taxa involved and direction of*  
 899 *introgression across five simulation conditions.*

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905 *Supplementary Figure 4: Accuracy of three methods (color legend) for estimating the rate of*  
 906 *introgression (y-axis) across four simulation conditions (x-axis). The horizontal dashed line*  
 907 *shows the true simulated rate of introgression.*

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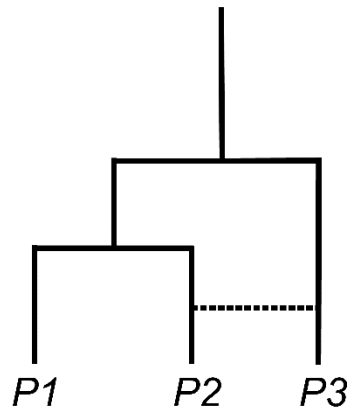
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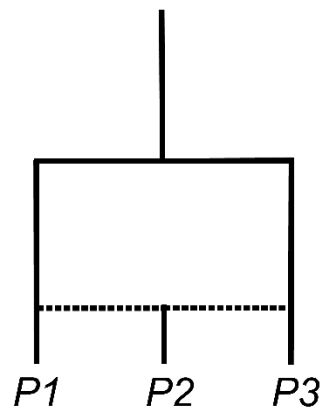
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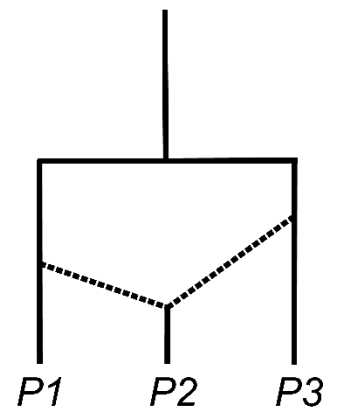
A)



B)



C)



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923 *Supplementary Figure 5: Network representations of introgression between extant lineages (A)*  
924 *vs. introgression that results in the formation of a new lineage (B, C).*

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Condition	P1/P2_split	P1P2/P3_split	P1P2P3/O1_split	O1/O2_split	intro_timing	intro_rate	ghostpop_split	theta
P1 into P3	0.6	1.2	8	20	0.3	0.05	N/A	0.005
P3 into P1	0.6	1.2	8	20	0.3	0.05	N/A	0.005
Sister species	0.6	1.2	8	20	0.3	0.05	N/A	0.005
Ancestor into P3	0.6	1.2	8	20	0.9	0.05	N/A	0.005
P3 into ancestor	0.6	1.2	8	20	0.9	0.05	N/A	0.005
High ILS	0.6	0.62	8	20	N/A	0.05	N/A	0.005
P1 into ingroup ghost	0.6	8	20	30	0.3	0.05	1.2	0.005
Ingroup ghost into P1	0.6	8	20	30	0.3	0.05	1.2	0.005
P1 into outgroup ghost	0.6	1.2	8	30	0.3	0.05	20	0.005
Outgroup ghost into P1	0.6	1.2	8	30	0.3	0.05	20	0.005

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944 *Supplementary Table 1: Parameters used for introgression simulation conditions in ms. Split*  
945 *times and theta are in units of 2N generations.*

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