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3 **Rapid and recent allopatric speciation in firs from central Mexico: evidence**
4 **for reinforcement?**

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20

21Abstract

22Secondary contact of species that have evolved partial reproductive isolation in
23allopatry may result in several outcomes, which range from rampant hybridization
24to barrier reinforcement. Reinforcement arises from reduced hybrid fitness, which
25promotes assortative mating and hence speciation. In plants, self-fertilization and
26disjunctions in reproductive-phenology are often invoked as evidence of
27reinforcement. However, local adaptation and pleiotropic effects during colonization
28can also lead to reproductive isolation without reinforcement. We explored these
29possibilities in a fir species complex (*Abies flinckii* - *A. religiosa*) distributed in 'sky-
30islands' along the Trans-Mexican Volcanic Belt (TMVB), in central Mexico. Despite
31co-occurring in two independent sympatric regions (west and center), these two
32taxa seem to rarely interbreed because of disjunct reproductive phenologies. We
33genotyped 1,147 SNPs, generated by GBS across 23 populations, and compared
34multiple demographic scenarios, built based on the geological history of the TMVB.
35The best-fitting model suggested a recent species split (for a conifer), dating back
36to ~1.2 Ma, together with early asymmetric gene flow (mostly from *A. flinckii* into *A.*
37*religiosa*), limited to the central sympatric region. Coupled with the lack of support
38for colonization models, the summary statistics (f , H_{obs} , F_{ST} , θ_{π} , etc.) and historical
39demographic inferences made herein point to a rapid speciation with an early
40development of reinforcement, as a putative mechanism for avoiding hybridization.
41The role of reinforcement should be thus further explored in the (sub)tropics, as
42likely explanation for how species diversity is generated and maintained.

43

44INTRODUCTION

45Speciation is a continuous process during which populations become
46reproductively isolated (Coyne & Orr, 2004; [Ravinet et al., 2017](#)). The evolution of
47reproductive barriers can be highly determined by the spatial distribution of
48populations (Coyne & Orr, 2004; [Gavrillets, 2003](#)). For instance, when they are
49separated by physical barriers that hamper gene flow, phenotypic and genetic
50differences are expected to evolve through the action of drift and/or divergent
51selection, which may ultimately contribute to reproductive isolation (Coyne & Orr,
522004; [Feder, Flaxman, Egan, Comeault & Nosil, 2013](#); Mayr, 1963). Conversely,
53speciation is thought to slow down as genetically distinct populations exchange
54alleles in the absence of physical barriers, or when they meet after evolving in
55geographic isolation for a period of time (i.e., secondary contact) ([Bolnick &](#)
56[Fitzpatrick, 2007](#), [Pinho & Hey, 2010](#)). Thus, if reproductive isolation is weak, as for
57recently diverged species, interspecific mating can still occur, and lead to stable
58hybrid zones ([Gompert, Mandeville & Buerkle 2017](#); [Payseur, 2010](#)).

59 The evolutionary outcome of a secondary contact zone depends in part on
60the fitness of the hybrid offspring. For instance, hybridization can spread if hybrids
61show high fitness, generating genetic introgression and leading to an eventual
62hybrid swarm (e.g., [Wells et al., 2019](#)). On the other hand, if hybrids have low
63viability and/or fertility, prezygotic barriers to gene flow are favored, and selection is
64reinforced for minimizing the high cost of interspecific mating ([Dobzhansky, 1940](#);
65[Servedio & Noor, 2003](#)). The process of reinforcement might involve the evolution
66of assortative mating, gametic incompatibilities, decoupling of breeding times
67and/or of reproductive morphology ([Hopkins, 2013](#)); thus, triggering stronger

68reproductive isolation in secondary contact regions, when compared to the non-
69overlapping portions of the parental species (e.g., [Rosser *et al.*, 2019](#), but also see
70Templeton, 1981).

71 In plants, the disjunction of reproductive phenologies and the evolution of
72selfing are known to prevent interspecific mating among sympatric related
73lineages, and are accordingly thought to play an important role during
74reinforcement ([Hopkins, 2013](#); e.g., [Fishman & Wyatt, 1999](#); [Osborne *et al.*, 2020](#);
75[Petit, Lesbros, Ge & Thomson, 1997](#)). However, selection against hybrids, and
76further reinforcement, can be confounded with other factors like historical
77demographic changes and/or local adaptation, which can also affect several traits,
78including mating system ([Gavrillets & Boake, 1998](#); [Noor, 1999](#); [Sottas, Reif,](#)
79[Kuczyński, & Reifová, 2018](#)). For instance, high dispersion capabilities and selfing
80are expected to be favored once a small number of individuals colonize a new
81habitat ([Knight *et al.*, 2005](#); [Pannell, 2015](#)). Under such a demographic context,
82assortative mating might also evolve by pleiotropic effects; for instance, when the
83alleles shaping the founders' reproductive phenology lead to local adaptation to the
84new environment ([Thibert-Plante & Gavrillets, 2013](#)). Therefore, the inference of
85historical demography and phylogeography is a crucial step for correctly identifying
86which of the aforementioned processes have led to the reduction of interspecific
87gene flow during secondary contact.

88 By assessing the potential of historical demography to explain reproductive
89isolation, we addressed this issue in a fir species-complex endemic to the
90mountains of central Mexico (i.e., the Trans-Mexican Volcanic Belt; TMVB). The
91TMVB is an east-west oriented volcanic formation (Figure 1), where fir forests are

92commonly distributed in ‘sky-island’ populations between ~ 2,000 and 3,500 m asl
 93(Rzedowski, 2006; [Velázquez, et al., 2000](#)). At least two species have been
 94recognized in this region: *Abies flinckii* and *A. religiosa* (Farjon & Rushforth, 1989).
 95They are respectively located in the western and central-eastern areas of the
 96TMVB (Figure 1). Morphologically, they can be differentiated by stem height and
 97diameter (larger in *A. religiosa*), color and shape of female cones (wider and darker
 98in *A. religiosa*), and needle length and apex form (longer needles with emarginated
 99apex in *A. flinckii*; shorter needles with acute apex in *A. religiosa*) ([Cruz-Nicolás et](#)
 100[al., 2020a](#); Eckenwalder, 2009; Farjon & Rushforth, 1989; Shalisko, 2014).
 101Distribution of these species partially overlap in two contact zones in the central
 102and western portions of the TMVB (respectively ‘contact C’ and ‘contact W’ in
 103Figure 1), although partially segregating by altitude, with *A. religiosa* occurring at
 104higher elevations (~2,800-3,500 m asl) than *A. flinckii* (~1,700-3,000 m asl)
 105(Rzedowski, 2006; [Vázquez-García, Shalisko, Cuevas-Guzmán, Muñiz-Castro &](#)
 106[Mantilla-Blandón, 2014](#)). Further complicating matters, the western-most
 107populations of the TMVB were recently recognized as another closely related
 108species of this complex: *A. jaliscana* ([Vázquez-García et al., 2014](#)). Nevertheless,
 109these populations are apparently isolated and not implicated in any sympatric zone
 110with the other two taxa. Interestingly, none of these species have a strongly
 111differentiating ecological niche (Cruz-Nicolás et al., 2020b; Martínez-Méndez,
 112Aguirre-Planter, Eguiarte & Jaramillo-Correa, 2016), although *A. flinckii* and *A.*
 113*religiosa* have mostly non-overlapping pollen phenologies (February-April in *A.*
 114*religiosa* vs. April-June for *A. flinckii*; Mantilla-Blandón 2006; [Vázquez-García et al.,](#)
 115[2014](#)), which is thought to prevent interspecific mating. This is reflected in clear

116morphological and nuclear genetic differentiation across contact W ([Cruz-Nicolás](#)
117[et al., 2020a](#); Shalisko, 2014). The other region, contact C, remains less studied
118and differentiation does not seem as clear.

119 While phenological differences between these firs might be the result of
120reinforcement, or character displacement, the geological context of the TMVB also
121provides a complex geological background for non-adaptive forces to drive such
122changes, as it would have facilitated conifer forest expansions in more than one
123occasion (Mastretta-Yanes, Moreno–Letelier, Piñero, Jorgensen & Emerson,
1242015). To elucidate which of the aforementioned process could better explain the
125phenological differences that apparently maintain species identities in contact
126zones, we evaluated the fit of several demographic scenarios on high-throughput
127genomic data with model-based coalescent analyses. These scenarios were based
128on the relatively well-known geologic and climatic history of the TMVB (Caballero-
129Miranda, Lozano-García, Vázquez-Selem & Ortega, 2010; Ferrari, Orozco-
130Esquivel, Manea & Manea, 2012; see below), which allow for both stochastic and
131non-stochastic evolution.

132 Our particular goals were a) to infer the potential for self-fertilization across
133populations using inbreeding coefficients, and test whether stronger genetic
134differentiation occurs in sympatric than in allopatric populations; b) to reconstruct
135the historical demography of this fir complex by testing competing long-distance
136colonization and fragmentation hypotheses; and c) to identify whether species
137divergence has occurred despite episodic gene flow events during secondary
138contact.

139 Our results settle a geologically-based demographic background for fir
140 evolution and suggests that reinforcement might have played a key role for
141 promoting rapid speciation. This is particularly important for understanding how
142 species diversity arises and is maintained in the (sub)tropics, and for slowly-
143 evolving lineages, like conifers.

144

145 MATERIALS AND METHODS

146 We collected foliar tissue for 123 trees from 23 populations spanning most of the
147 known fir distribution across the TMVB (Figure 1, see Aguirre-Planter, Furnier &
148 Eguiarte, 2000; Méndez-González, Jardón-Barbolla & Jaramillo-Correa, 2017 for
149 additional sampling details). Genomic DNA was isolated with a CTAB 2x mini-prep
150 protocol (Vázquez-Lobo, 1996); its quality was evaluated by agarose gel
151 electrophoresis using a 1,000 bp DNA marker as reference (Thermo Scientific™),
152 and its concentration quantified with a Qubit 4 fluorometer (Thermo Scientific™).

153

154 *Genotyping by sequencing (GBS), assembly and SNP calling*

155 Following standard protocols for plants with large and complex genomes
156 ([Boudhrioua et al., 2017](#); [Poland, Brown, Sorrells & Jannink, 2012](#)), genomic DNA
157 (200 ng per sample) was first double-digested with *Pst*I and *Msp*I restriction
158 enzymes. Then, P1 adapters containing 4- to 8-bp-long individualized barcodes
159 (differing from each other by at least 2 bp) were ligated to each sample. Equimolar
160 quantities of DNA were pooled to elaborate GBS libraries after sizing with a blue
161 Pippin prep (SAGE sciences, USA) machine. Each 96-plex library was single-end-

162sequenced on an Illumina HiSeq 2500 system. Both GBS library preparation and
163sequencing were performed at the Plateforme d'analyses génomiques, of the
164Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval, Québec,
165Canada).

166 Raw reads filtering, parameter optimization, contig assembly and SNP
167calling were performed following the ipyrad pipeline (Eaton & Overcast, 2020).
168Briefly, after demultiplexing, Illumina adapters and low quality 3' ends were
169trimmed (Phred score < 43). We kept only those reads that lacked low quality
170bases and had a minimal length of 80 bp. Then, we performed a *de novo assembly*
171using Vsearch v.2.0.2 (Rognes, Flouri, Nichols, Quince & Mahé, 2016), with
172parameters defined after optimization, using 11 replicate pair samples (Table S1,
173Figure S1, see Supplementary Material 1). We considered a clustering threshold of
1740.85, a max number of 4 heterozygous sites per locus (i.e., contig), and a minimum
175statistical depth of 8 reads. However, only those loci exhibiting a single SNP were
176considered, as these are the least prone to be spurious polymorphisms (Figure S1
177b and c, see Supplementary Material 1). Such a stringent filter diminishes LD bias
178between SNPs in population genomic analyses ([Zheng et al., 2012](#)). Singletons
179(i.e., mac= 2), indels, non-biallelic SNPs and polymorphisms with a mindepth of
18010x or less, and 100X or more were removed using Vcftools v. 0.1.14 (Danecek et
181al., 2011). As an additional filter, retained SNPs were compared with those
182obtained from a sequenced *A. religiosa* megagametophyte (the haploid tissue
183surrounding conifers embryos). Those observed in both datasets were eliminated
184as they are the likely result of paralog misalignment.

185 We finally constructed three datasets by excluding sites with 50%, 13% and
1860% missing data, respectively, to identify potential bias in our inferences. These
187datasets were used to model some genomic diversity statistics as a function of
188missing data level, and to account for this factor during demographic model
189selection. After verifying that missing data were not producing serious confounding
190effects (see Supplementary Material 2), the 50% missing-data set was used as
191input for all subsequent analyses.

192

193*Potential to self-fertilize, genomic diversity and inbreeding*

194As a proxy to self-fertilization, we first estimated population inbreeding coefficients
195(f) from an unbiased kinship matrix constructed with R package *popkin* v. 1.2.2
196(Ochoa & Storey, 2016), which was adjusted for the most likely genetic structure
197according to ADMIXTURE analyses (see below). We then computed the ratio of
198heterozygous to segregating sites (Het/S), the average number of nucleotide
199differences (θ_{π} ; Tajima, 1983), the nucleotide diversity (θ_w ; Watterson, 1975), the
200observed heterozygosity (H_{obs}) and Tajima's D (Tajima, 1989) using DnaSP v. 6
201(Rozas *et al.*, 2017) and Vcftools v. 0.1.14 (Danecek *et al.*, 2011). Under the
202reinforcement hypothesis, increased levels of inbreeding and diminished values of
203genomic diversity are expected in sympatric populations when compared to
204allopatric stands ([Charlesworth & Pannell, 2001](#); e.g., [Rosser *et al.*, 2019](#)), while
205such values should be homogenous under the pleiotropic hypothesis. We
206additionally calculated pairwise F_{ST} (Hudson, Slatkin & Maddison, 1992) in
207PopGenome v. 2.7.1 (Pfeifer, Wittelsb rger, Ramos-Onsins & Lercher, 2014) to
208ask for significantly higher differentiation in secondary contact than in allopatric

209 zones, which would suggest stronger barriers to gene flow in the first areas,
210 pointing to reinforcement.

211

212 *Genetic structure*

213 To detect population genetic structure, we conducted a PCA with SNPRelate
214 ([Zheng et al., 2012](#)), and a model-based assignment using ADMIXTURE v.1.3.0
215 (Alexander, Novembre & Lange, 2009). For this last method, we performed ten
216 independent runs, each one with *K*-values ranging from one to eight; the *K*-value
217 with the lowest averaged cross-validation error was chosen as the most likely.
218 Since westernmost populations (i.e., populations 1-10) showed high inbreeding
219 coefficients (see Results), we repeated ADMIXTURE analyses without these
220 localities. We finally carried out a Discriminant Analysis of Principal Components
221 (DAPC) for this reduced population set, with R package adegenet (Jombart, 2008),
222 for identifying more subtle differences between and within clusters, including
223 isolation by distance (Jombart, Devillard & Balloux, 2010). The most likely number
224 of PCs was investigated with a cross-validation procedure implemented also in
225 adegenet.

226

227 *Historical range shifts of firs in the TMVB*

228 Besides reinforcement, other processes such as founder events during the
229 colonization of new environments could result in the evolution of prezygotic
230 reproduction barriers (Pannell, 2015). To test this possibility, we fitted several
231 divergence and demographic scenarios to the 'observed' multi-site frequency
232 spectrum (MSFS). We conducted a hierarchical comparison of scenarios to

simplify hypothesis testing. Hence, we first focused on evaluating three divergence scenarios at the species level (see Figure S4), which respectively involved ancient colonization events (i.e., Ha and Hb scenarios) and a more recent divergence driven by fragmentation (i.e., Hc scenario). Then, the best-fitted scenario was used as a baseline to test four more complex hypotheses at the population /regional levels. These explored in more detail intraspecific range-shifts within *A. religiosa*, considering either one (i.e., Hc9, Hc10 scenarios) or two source populations (i.e., Hc11 and Hc12) Figure S5.

The geological evolution of the TMVB has been characterized by the uplifting of four temporal and spatially localized magmatic arcs (Ferrari *et al.*, 2012), together with some local topographic modifications, like the rising of tectonic valleys in its western portion ([Rosas-Elguera *et al.*, 2003](#)). Thus, all demographic scenarios were parameterized considering colonization events from older to younger arcs; and/or population splits between rising tectonic valleys (Figure S3). A detailed description of each demographic model can be found in Supplementary Material 3; 'priors' for divergence time parameters are available in the Table S3.

We derived 'observed' MSFS from the 50% missing-data SNP dataset with the script easySFS.py ([Overcast, 2019](#)). This MSFS was then fitted to those produced by each demographic scenario. Scenarios were simulated in fastsimcoal2 v 2.5.2 (Excoffier, Dupanloup, Huerta-Sánchez, Sousa & Foll, 2013), and consisted of 100 independent runs of 150,000 coalescent iterations each. The run with the best likelihood for each scenario was used to estimate AIC weights (w_{AIC} , [Burnham & Anderson, 2002](#)) and select the best-fitting demographic model. Since the coalescent assumes random individual mating, highly inbred populations/

species such as those from the western TMVB may have led to inaccurate demographic parameters (Blischak, Barker & Gutenkunst, 2020). To take this into account, we further included observed inbreeding values as fixed parameters for all western gene pools (i.e., *A. flinckii*, *A. jaliscana* and W stand).

261

262 *Interspecific mating*

Under reinforcement, prezygotic barriers to reproduction are expected to evolve as a response to maladaptive offspring resulting from interspecific gene flow (Hopkins, 2013). Thus, if this hypothesis is correct, the most supported demographic scenario should include interspecific gene flow, but only at early stages of species divergence. To test this, we compared the retained models above (no gene flow) with models including ancient gene flow (i.e., shortly after species split), and more recent migration (Figure 4). Such recent events were associated with the mid-late Pleistocene glacial periods, which would have favored population down-slope migration and expansion; thus, increasing chances for secondary contact and gene flow (Figure S3 c; Caballero-Miranda *et al.*, 2010; Ramírez-Barahona & Eguiarte, 2013). All models allowed for asymmetric gene flow between neighboring species/genomic groups.

To compare and select the best-fitting scenario(s), we implemented the same aforementioned strategy in fastsimcoal2. For each chosen model, we first used the maximum likelihood estimates to simulate 100 parametric bootstrapped MSFSs and calculate 95% confidence intervals for population parameters. Then, for each MSFS, we recovered the best-fitted population parameters from a total of 15 independent likelihood runs to finally generate distribution parameters.

281 Divergence times were transformed into million years by assuming a generation
282 time of 60 years (Sánchez-Velásquez, Pineda-López & Hernández Martínez,
283 1991).

284

285 RESULTS

286 GBS and bioinformatic analysis

287 Approximately 260 million reads of about 100 bp, and with a mean Phred score of
288 36, were obtained per sequencing lane. After SNP calling and filtering, 1,147, 602
289 and 138 SNPs were respectively recovered when allowing for only one SNP per
290 loci and 50%, 13% and 0% missing data. Each polymorphism had a mean depth
291 sequencing of 52 across individuals. Linear mixed models indicated only a weak
292 effect of missing data on some genomic statistics, like inbreeding coefficients (f)
293 and observed heterozygosity (H_{obs}) (Table S2, Figure S2, see Supplementary
294 Material 2), thus we used the SNP dataset with 50% missing data for all remaining
295 analyses.

296

297 Inbreeding coefficients and genomic diversity

298 Inbreeding coefficients (f) were higher for *A. flinckii* ($f = 0.592 - 0.828$) and *A.*
299 *jaliscana* ($f = 0.575 - 0.648$) than for *A. religiosa* ($f = 0.302 - 0.733$; $t = -9.214$, P -
300 value = $2.3e^{-15}$; Figure 1; Figure S2). Such values were further significantly higher
301 at contact W ($t = 5.949$, P -value = $3.2e^{-08}$; Figure 1) than in the rest of populations,
302 including those from contact C. Consistently, lower Het/S ratios ($t = 3.570$, P -value
303 = 0.001918), H_{obs} ($t = 9.214$, P -value = $2.3e^{-15}$), θ_{π} ($t = 4.180$, P -value = 0.000462 ;
304 Figure 1) and θ_w values ($t = 4.571$, P -value = 0.000185) were observed for western

305fir species than for *A. religiosa* (Figure 1, Figure 2a, Table S4). Again, these
306estimates were significantly lower for contact W than for contact C or all allopatric
307stands ($t_{\text{He/S}} = -2.789$, $P\text{-value} = 0.0113$; $t_{\text{Hobs}} = -5.949$, $P\text{-value} = 3.2\text{e-}08$; $t_{\text{ew}} = -$
3083.317, $P\text{-value} = 0.00344$; $t_{\text{PI}} = -3.514$, $P\text{-value} = 0.00218$; Figure 1, Figure 2a,
309Table S4).

310 Furthermore, *A. flinckii* and *A. jaliscana* showed positive Tajima's D , while
311for *A. religiosa* such values were negative, excepting those from populations at
312contact W, which were positive (Table S4). Higher pairwise F_{ST} were observed
313between- than within-species ($t = -27.23$, $P\text{-value} < 2\text{e-}16$; Figure 2b), excepting for
314*A. flinckii* ($F_{ST} = 0.330$; $\text{sd} = 0.148$). Sympatric population pairs of *A. flinckii* and *A.*
315*religiosa* within contact W had higher F_{ST} values than allopatric pairs ($F_{ST} = 0.597$
316vs. 0.508 ; $t = 2.599$, $P\text{-value} = 0.011$), while pairs within contact C had virtually
317identical F_{ST} values than allopatric pairs ($F_{ST} = 0.491$, $\text{sd} = 0.047$) (Figure 2b).

318

319Genetic structure

320The first two axes of the PCA explained 7.7 and 6.3% of the genomic variance,
321respectively, and separated individuals according to taxonomy, including those
322recently described as *A. jaliscana* (Figure S6 a). ADMIXTURE mirrored these
323results, with a retained $K\text{-value}$ of 3 (Figure 3c). However, further genomic
324structure was revealed for higher K values (Figure S6 c). For $K= 4$, *A. religiosa*
325populations from contact W separated in an independent cluster (i.e., W group).
326For $K= 5$, the rest of *A. religiosa* individuals were divided along an east-to-center
327genetic cline (E and C groups, respectively), with some admixture observed in
328populations 12, 13 and 14 (grouped hereafter in "M" group for within-species

analyses, Figure 3c). Unexpectedly, the northernmost population (i.e., Pop 23) was clustered within the C group (Figure 3a). Finally, for $K=6$, the two easternmost populations (i.e., Pops 19 and 20) were separated from the rest (P group); some individuals from populations 21 and 22 further showed introgression from this group (Figure 3c).

After excluding the highly inbred western populations (i.e., *A. jaliscana*, *A. flinckii* and W stands of *A. religiosa*), no genetic structure was observed with ADMIXTURE (Figure S6 d). However, DAPC analyses did show a similar intraspecific genetic structure for *A. religiosa* than the one previously observed for the whole dataset with ADMIXTURE, supporting both P and W clusters, and the genetic cline between E and C groups (Figure 3b).

Inter and intraspecific divergence

At the species level, the best-fitting model (according to likelihood and $wAIC$ estimations; Table S5) was Hc, which consisted of a recent species divergence, likely driven by the arising of tectonic valleys (Figure S4 c). Similar results were obtained when using datasets with less missing data, and after representing *A. religiosa* by just the C or E genetic pools (Table S5); thus, population substructure or missing data should not be affecting model choice at this level (Table S5).

Model Hc was then used as base line for testing four more complex demographic hypotheses (Figure S5), and address population divergence within *A. religiosa*. According to AIC and $wAIC$, scenarios Hc9 and Hc12 were equally likely for explaining within-species genetic structure (Table S6). The former comprised a serial colonization from east to west (i.e., Hc9 in Figure 4), while the latter included

the initial divergence of two gene pools (C and E), followed by their expansion and secondary contact (i.e., Hc12 in Figure 4). These two scenarios were retained for testing for interspecific mating by integrating gene flow at different times.

356

Incorporating gene flow and estimating parameters

The highest $wAIC$ was obtained when ancient interspecific gene flow (i.e., shortly after species split) was incorporated into scenario Hc12 (two ancestral gene pools within *A. religiosa*; Figure 5; Table 1), which further refutes the serial colonization hypothesis. According to this scenario, the divergence between *A. religiosa* and the western firs occurred ~1.26 Ma ago (95% CI: 1.25-1.27 Ma), and was followed by the split of E and C genomic pools within *A. religiosa*, which was dated to ~1.06 Ma (95% CI: 1.03-1.08 Ma). The divergence between *A. flinckii* and *A. jaliscana* was estimated in ~0.402 Ma (95% CI: 0.37-0.43 Ma) (Figure 5). Within *A. religiosa*, the range expansion of E and C genetic pools, and the differentiation of W and P groups, would have taken place ~0.218 Ma ago (95% CI: 0.214-0.221 Ma (Table 2; Figure 5).

Inferred ancestral effective population sizes (N_e s) were large for all cases, except for the ancestor of western firs (Table 2, Figure 5), which had a severe decline after divergence. Modern N_e (after accounting for inbreeding) for *A. flinckii* and *A. jaliscana* suggested a subtle recovery after that initial collapse. On the other hand, ancestral *A. religiosa* genetic pools had large N_e s, although estimates for modern lineages suggest population decline; such modern N_e s are all in the same order of magnitude than those estimated for the western firs. Other than occurring early after species divergence, gene flow was apparently highly asymmetrical, with

377most migration taking place from western firs into *A. religiosa* (i.e., C genetic
378group). Gene flow was also higher from *A. flinckii* into *A. jaliscana* than in the
379opposite direction (Figure 5; Table 2).

380

381DISCUSSION

382Disentangling the drivers and microevolutionary processes underlying prezygotic
383reproductive isolation remains a challenge, especially for non-model systems, like
384conifers (Campbell, Poelstra & Yoder, 2018). Here, we addressed this in a fir
385species complex from central Mexico, by comparing patterns of genomic variation
386between allopatric and sympatric zones, together with past demographic
387inferences. Our models suggest that species diverged in the presence of ancient
388interspecific mating, and show no sign of recent gene flow (i.e., after secondary
389contact). Our simulations suggest that the reported differences in reproductive
390phenology did not originate after founder events during range expansions; instead,
391they likely appeared early on after species divergence.

392

393*Rapid species differentiation and evolution of inbreeding tolerance in western firs*
394Our approximation, with a much larger number of markers and a more complete
395battery of analyses than in previous attempts (e.g., Aguirre-Planter *et al.*, 2000,
396Jaramillo-Correa *et al.*, 2008), revealed well-defined genetic clusters with limited
397admixture, even in secondary contact areas (Figure 3a and 3c). Such a structure
398coincides with the most recent taxonomic descriptions for central Mexican firs

399(Figure 1 and 3a), including the recognition of the westernmost populations as a
400different species, *Abies jaliscana* (Vázquez-García *et al.*, 2014).

401 The formation of the TMVB started in the early Miocene and included the
402uplifting of four main volcanic arcs (Ferrari *et al.*, 2012). Further changes occurred
403later-on, during the late Pliocene and the early Pleistocene, through fragmentations
404in the western portion driven by the formation of large tectonic valleys ([Rosas-](#)
405[Elguera *et al.*, 2003](#); Figure 2a and 5b). Such landscape consists of largely
406separated mountains with isolated temperate forests in their tops (i.e., sky-islands),
407which would have promoted the allopatric divergence of species/populations
408(Mastretta-Yanes *et al.*, 2015; [Ramírez-Barahona & Eguiarte, 2013](#)). Such isolation
409is reflected in the small ancestral N_e estimated herein for the western firs. While
410population expansions were inferred at the local scale after species divergence
411(i.e., for *A. flinckii* and *A. jaliscana*), N_e s remained small when compared to *A.*
412*religiosa* as a whole (i.e., all genetic groups combined; Figure 5).

413 Our demographic analyses further support that interspecific gene flow only
414occurred shortly after species divergence, and not after secondary contact (Figure
4155), which indicates that *A. flinckii* and *A. jaliscana* have likely remained isolated for
416long periods of time. Both genetic isolation and reduced N_e s should have prompted
417rapid stochastic evolution, leading to the fixation of genetic variants by genetic drift
418(Ohta, 1992; Lanfear, Kokko & Eyre-Walker, 2014), as evidenced by their generally
419low genetic diversity and positive Tajima's *D* values (Figure 1 and 2a, Table S4).
420Such values are particularly noteworthy when compared to those of *A. religiosa*
421and more northern conifers (Johnson, Gaddis, Cairns, Konganti & Krutovsky, 2017;
422Menon *et al.*, 2018; Elleouet & Aitken, 2019), which are more genetically diverse.

423 The historical demography of western firs further hints several worth-testing
424hypotheses for future studies that use controlled crosses and an extensive coding-
425gene coverage. For instance, the observed inbreeding for both *A. flinckii* and *A.*
426*jaliscana* suggests that some selfing tolerance may have evolved as an adaptive
427strategy to small population sizes (Restoux *et al.*, 2008). Like for other conifers,
428selection at early life stages might have helped western firs purge most of the
429highly deleterious variation, as expected for populations with small N_e s (Ferriol,
430Pichot & Lefèvre, 2011; Restoux *et al.*, 2008), and recently observed for a
431genetically depauperated pine (Jaramillo-Correa *et al.*, 2020). Likewise, such a
432demographic background might have promoted the accumulation of slightly
433deleterious mutations, some of them putatively involved in hybrid genetic
434incompatibilities (i.e., Bateson-Dobzhansky-Muller incompatibilities, BDMIs;
435[Matute, Butler, Turissini & Coyne, 2010](#); Maya-Lastra & Eaton, 2021; Orr & Turelli
4362001; [Widmer, Lexer & Cozzolino, 2008](#)). Looking for such incompatibilities should
437be thus a logical next step for understanding rapid conifer evolution in the
438(sub)tropics.

439

440*Early interspecific gene flow at contact C: evidence for reinforcement?*

441The evolution of prezygotic barriers, including shifts in reproductive phenology, are
442favored by selection when hybrids suffer from lower fitness than parental species,
443which reinforces speciation after secondary contact (Hopkins, 2013; Servedio &
444Noor, 2003). Our results indicate that the differential pollen phenologies between
445*A. flinckii* and *A. religiosa* are indeed preventing interspecific mating (Figure 3a),
446especially in contact W. In this region, the reduced genomic diversity, and the

447increased inbreeding and genomic differentiation, suggest a stronger assortative
448mating and reproductive isolation than in contact C, where populations exhibited
449similar genetic diversity and inbreeding coefficients than in allopatric stands (Figure
4501 and 2; Table S4). A priori, this would point to differential demographic/selective
451pressures between contact regions, with evidence for reinforcement only in the
452western zone (contact W). However, our demographic inferences indicate that
453interspecific gene flow (which allows for reinforcement to occur) only took place at
454the earlier stages after species divergence, exclusively in the central portions of the
455TMVB (i.e., purple polygon in Figure 3a), and before the isolation of the W stands
456(i.e., red triangle in Figure 5).

457 While the signature of reinforcement might have eroded in contact C,
458because of the highly dynamic demographic history of *A. religiosa* in this region
459(Figure 5), the reinforcement pattern inferred in contact W could be actually the
460result of other demographic processes, like those incurred during founder events
461(Gavrilets & Boake, 1998; Noor, 1999; [Pannell, 2015](#)). However, our demographic
462analyses rejected the colonization hypotheses (i.e., Ha and Hb scenarios), and
463favored instead the ancestral divergence one (i.e., Hc scenario), which only
464included strong population fragmentation in the West (i.e., Hc, Table S5).

465 Another competing possibility is that local adaptation and ecological
466displacement promoted reproductive trait divergence (and speciation) in sympatry
467(Hopkins, 2013). However, since the species studied herein exhibit no climate
468niche differences (Martínez-Méndez *et al.*, 2016; Cruz-Nicolás *et al.*, 2020b), the
469role of such factors is unlikely. Indeed, theoretical models have shown that gene
470flow between diverging taxa with reduced ecological differences hampers the

471 evolution of reproductive isolation, like the aggregation of BDMLs (Blanckaert &
472 Hermisson, 2018; Blanckaert, Bank & Hermisson, 2020; [Xiong & Mallet, 2021](#)) or
473 the development of prezygotic barriers through reinforcement (Servedio &
474 Kirkpatrick, 1997). Such taxa would instead form stable hybrid zones, in which
475 speciation might take long periods of time to be completed. While most conifers fit
476 these expectations (Bolte & Eckert, 2020; e.g., in [Neale & Wheeler, 2019](#)), the
477 studied firs from central Mexico showed little signs of hybridization and, according
478 to our results, diverged (and reproductively isolated) at a much faster pace. This is
479 a real rarity within this group of plants. Indeed, to our knowledge, only *Juniperus*
480 *phoenicea* and *J. turbinata* share certain similarities with our study system, as their
481 phenological differences also prevent interspecific mating at natural contact zones
482 (Arista, Ortiz & Talavera, 1997).

483 Given that gene flow and hybridization only apparently took place at the
484 early stages after species divergence (Figure 5), it is difficult to directly estimate
485 the cost of interspecific mating during the evolution of reinforcement. However, our
486 best-fitted demographic scenario does suggest some expected outcomes for
487 eventual modern hybrids, which are worth studying in the future. For example, the
488 contrasting N_e values estimated herein point to a differential distribution of fitness
489 effects between species, with more strongly and mildly deleterious alleles
490 segregating in *A. religiosa*, probably as heterozygous and at low frequencies, than
491 in *A. flinckii*, which would have purged most of the strongly deleterious alleles. This
492 last species might, however, still have a significant portion of mildly deleterious
493 variants segregating at high frequencies (Lanfear *et al.*, 2014). If that is the case, a
494 successful interspecific mating would result in hybridization load, for instance

495through BDMIs (Harris & Nielsen, 2016; [Moran et al., 2020](#); [Ottenburghs, 2018](#);
496[Pickup et al., 2019](#)). However, testing this hypothesis will require much more
497developed genomic tools and controlled crosses (e.g., [Abadie et al., 2012](#); Stacy,
498Paritosh, Johnson & Price, 2016).

499 The observed differences in N_e between *A. flinckii* and *A. religiosa* could
500also explain the asymmetric gene flow inferred herein. That is, species/populations
501with smaller N_e are expected to have lower recombination rates than those with
502larger N_e (Stapley, Feulner, Johnston, Santure & Smadja, 2017, e.g., Lalagüe et
503al., 2014). Thus, hybrids back-crossed to *A. flinckii* would have larger linkage
504blocks than those to *A. religiosa*, and selection against those blocks that contain
505introgressed (mildly) deleterious alleles would result in lower global rates of
506introgression in the first direction than in the second one (see also Harris &
507Nielsen, 2016; Juric, Aeschbacher & Coop, 2016; Schumer et al., 2018). Again,
508more detailed genomic analyses would be necessary to test this hypotheses.

509

510*Abies religiosa* and diversification in subtropical montane systems

511Other than rapid species divergence, our demographic models suggest that *A.*
512*religiosa* expanded from the center of the TMVB towards both the east and the
513west during the late Pleistocene (~ 218 Kya, Figure 5). Such an expansion
514corresponds with the last formation phase of the TMVB, which included the
515uplifting of large stratovolcanoes (Ferrari et al., 2012), that now provide suitable
516habitats for fir forests (Velázquez et al., 2000; Rzedowsky, 2006). While the weak
517but significant genetic structure observed for *A. religiosa* (Figure 3b & c) might be

the result of geographic isolation during interglacial stages, as has been proposed for sky-island systems (Mastretta-Yanes *et al.*, 2015; Ramírez-Barahona & Eguiarte, 2013), it is interesting that our demographic analyses did not support recent gene flow between lineages (Table 1). Whether these exchanges occurred, as suggested by palynological data and niche projections into the Last Glacial Maximum (Caballero-Miranda *et al.*, 2010; Cruz-Nicolas *et al.*, 2020b), this would be a hypothesis to test at finer geographic scales and using a different sampling strategy than the one used herein.

Finally, it would be worth testing if the main role of geologic and topographic features in shaping N_e s and promoting rapid genetic divergence also applies for other (sub)tropical conifers. The possibility that incompatibilities can quickly get fixed in allopatry, by either drift or reinforcement, even between species prone to long-distance dispersal and with rather permeable reproductive barriers, could indeed explain the large conifer diversity, and that other temperate taxa like oaks, in (sub)tropical montane regions (Gernandt & Pérez-de la Rosa, 2014; Rahbek *et al.*, 2020; e.g., Moreno-Letelier, Mastretta-Yanes & Barraclough, 2014; Peláez, Ortiz-Martínez, Figueroa-Corona, Montes & Gernandt, 2020).

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879**DATA ACCESSIBILITY**

880Raw sequencing data and filtered variant SNP file used for population genomic

881analyses have been deposited at Dryad Repository under the accession doi:

882XXXXX (available upon acceptance). Detailed SNP calling procedure and sampling

883information can be found in the Supplementary section. Scripts used for analyses

884are available at <https://github.com/XXXX> (available upon acceptance).

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887**AUTHOR CONTRIBUTIONS**

888The study was conceived by G.I.G-P and J.P.J-C. Collection of samples was
889carried out by E.A-P. and G.I.G-P. Bioinformatic and statistical analyses were
890performed by GI G-P. GI G-P and JP J-C interpreted the results and drafted the
891manuscript; all co-authors reviewed it and approved its final version.

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Table 1. - Likelihood-based model choice for six demographic scenarios of fir divergence in central Mexico. Models consisted of either a serial colonization from one source or a range shift from two source populations. Three migrations patterns were further tested: no-migration, ancient gene flow and recent gene flow (see Figure 4). Best fitted model based on Akaike weight (w_{AIC}) in bold.

Model	Process	Migration	Likelihood	N _{Params}	Δl_{hood}	AIC	ΔAIC	w_{AIC}
Hc9	Serial colonization	No	-790.665	14	97.027	3669.147	19.771	4.159e ⁻⁰⁵
Hc12	2 pop sources	No	-789.946	16	96.308	3669.836	20.460	2.947e ⁻⁰⁵
Hc9	Serial colonization	Recent	-781.499	29	87.861	3656.936	7.560	1.865e ⁻⁰²
Hc12	2 pop sources	Recent	-780.120	30	86.482	3652.585	3.210	1.642e ⁻⁰¹
Hc9	Serial colonization	Ancient	-788.346	19	94.708	3668.467	19.092	5.842e ⁻⁰⁵
Hc12	2 pop sources	Ancient	-783.766	20	90.128	3649.376	0.000	8.171e⁻⁰¹

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Table 2.- Estimated population parameters that maximize the likelihood function of the best fitted demographic model for fir divergence in Central Mexico (Figure 5). Upper and lower 95% confidence intervals estimated after computing parameters from 100 bootstrapped MSFSs. M, migration rates. Divergence times (Tec, Or, Occ, Exp) were transformed to million years by assuming a generation time of 60 years (Sánchez-Velásquez *et. al.*, 1991).

Parameter	Upper	Mean	Lower
<i>A.jaliscana</i> (J)	6998	6622	6246
<i>A.flinckii</i> (F)	3731	3463	3196
<i>A.religiosa</i> (W)	4864	4627	4390
<i>A.religiosa</i> (C)	3714	2475	1236
<i>A.religiosa</i> (M)	2527	2451	2374
<i>A.religiosa</i> (E)	3469	3392	3315
<i>A.religiosa</i> (P)	3091	3025	2958
Ancient-West	89457	86792	84128
Ancient-East	77715	71527	65339
F-J ancestral pop	125	119	112
Fir ancestral pop	59243	54884	50525
Admixture	0.207	0.163	0.119
Tec	1.271	1.26	1.249
Or	1.082	1.056	1.03
Occ	0.43	0.402	0.373
Exp	0.221	0.218	0.214
$\dagger M_{F \leftarrow C}$	$1.27e^{-04}$	$1.02e^{-04}$	$0.76e^{-04}$
$\dagger M_{C \leftarrow F}$	$92.92e^{-04}$	$86.11e^{-04}$	$79.31e^{-04}$
$\dagger M_{J \leftarrow F}$	$12.30e^{-04}$	$8.08e^{-04}$	$3.88e^{-04}$
$\dagger M_{F \leftarrow J}$	$4.54e^{-04}$	$3.19e^{-04}$	$1.85e^{-04}$

902[†] arrows indicate the direction of gene flow

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Figure 1. Geographic location of the 23 fir populations studied along the TMVB, in central Mexico. Circles outlined in light brown and black correspond to *A. flinckii* and *A. religiosa*, respectively; those with dotted margins are populations described as *A. jaliscana* (Vázquez-García *et al.*, 2014). Circle size is proportional to the average number of pairwise differences between individuals (θ_π , Tajima, 1983), and color denotes the average inbreeding (f) coefficient per population (see scales on the right). Blue and red squares indicate the approximate location of secondary contact zones between *A. flinckii* and *A. religiosa*.

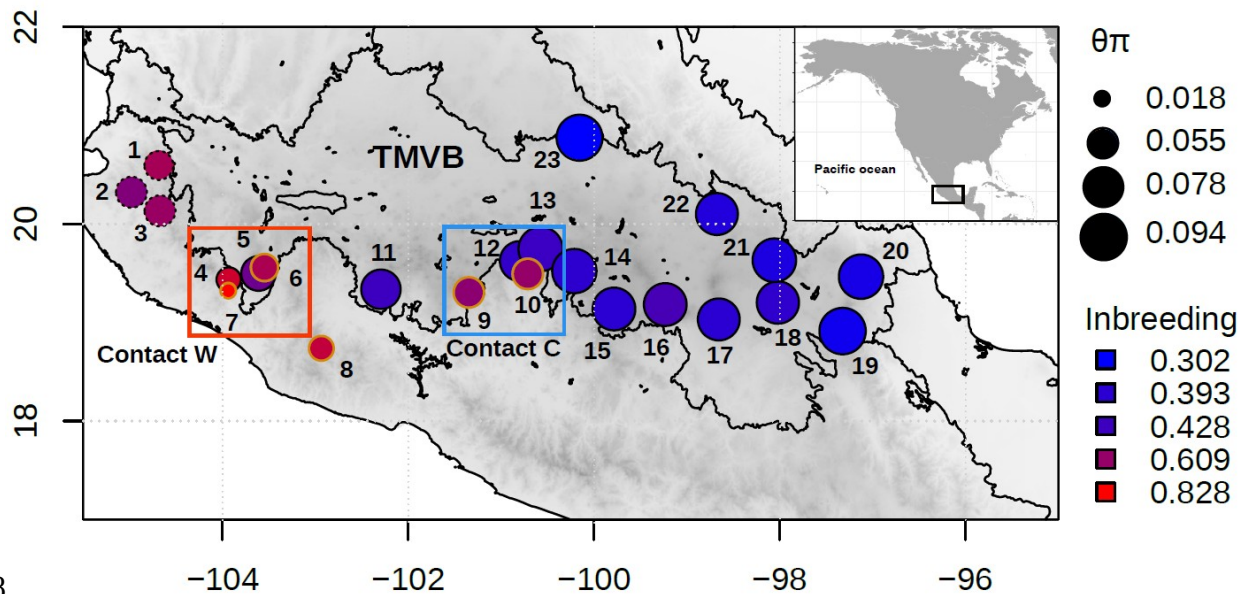


Figure 2. Boxplots of observed heterozygosity (H_{obs}) per species (**a**) and pairwise F_{ST} (Hudson, 1992) (**b**) between conspecific (*A.jalis*, *A.relig*, *A.flin*) and interspecific populations (J-R, F-R, J-F) in central Mexico. Red and blue points highlight H_{obs} and interspecific pairwise F_{ST} values within W (red) and C (blue) contact zones, respectively.

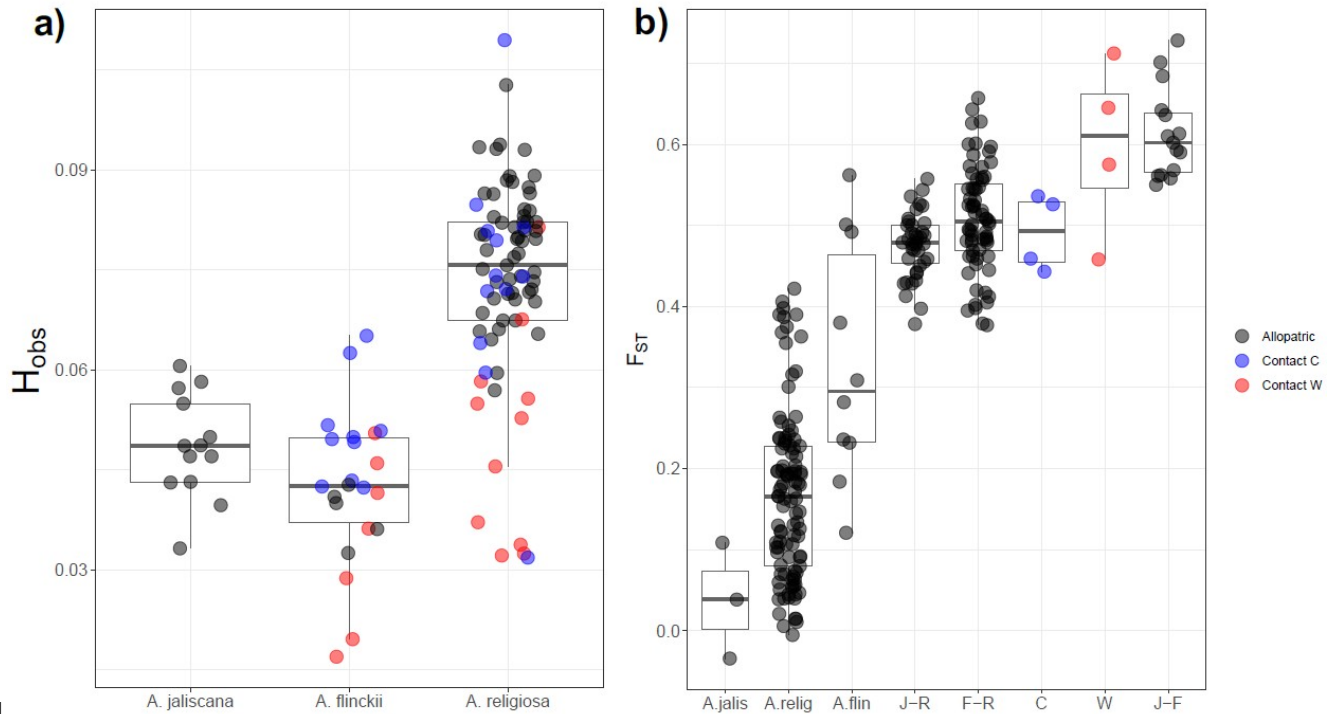
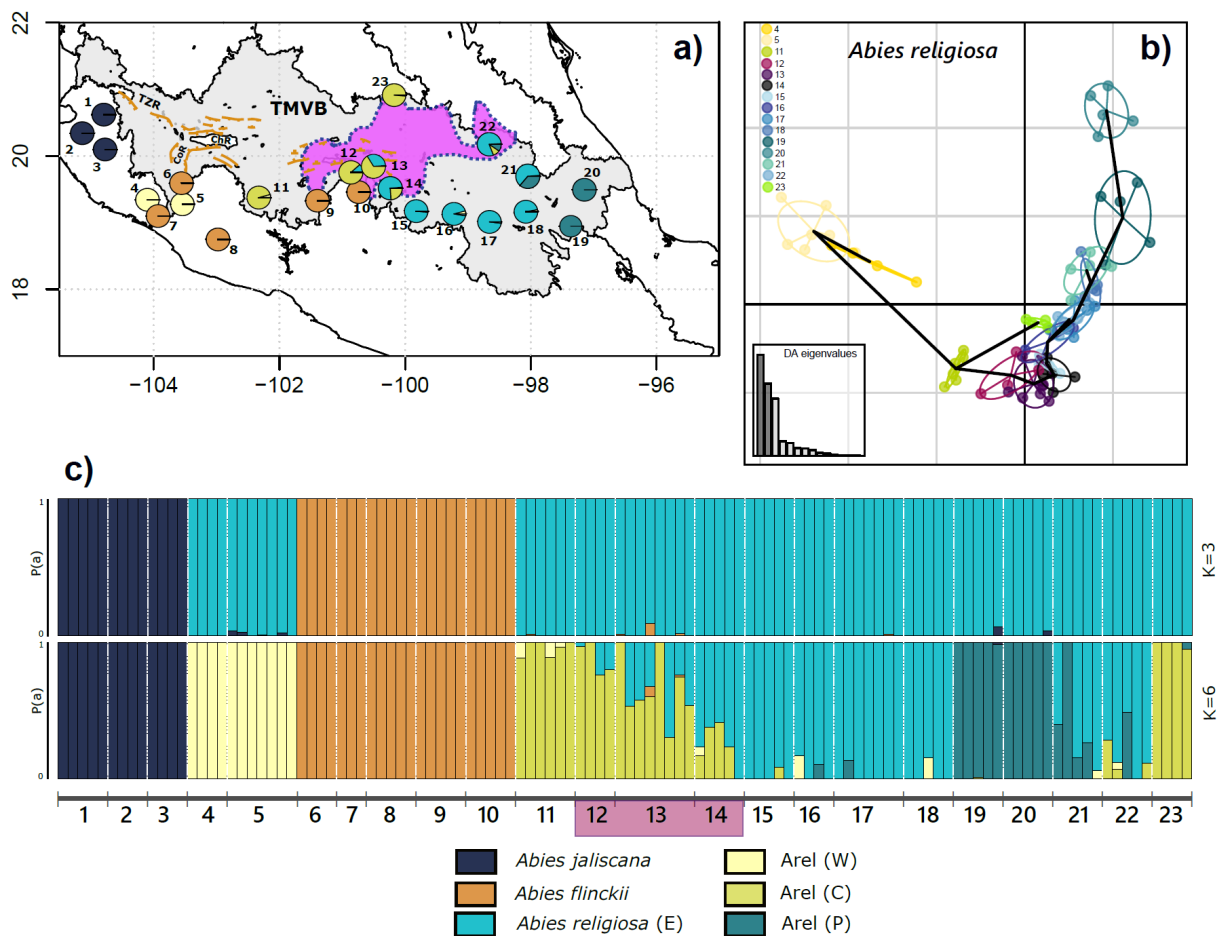


Figure 3. Population genetic structure of fir populations along the TMVB, in central Mexico. **a)** Geographic distribution of genetic ancestries when assuming $K=6$ in ADMIXTURE analyses (depicted in **c**). Brown lines illustrate the main tectonic valleys in the region (TZR, Tepic-Zoacalco rift; CoR, Colima rift; ChR, Chapala rift; Rosas-Elguera *et al.*, 2003). The purple region is the active volcanic landscape between 7.5 and 3 Mya (Ferrari *et al.*, 2012), where speciation likely took place (see Results). **b)** Discriminant analysis of principal components (DAPC) for *A. religiosa* populations. Black lines represent a minimum spanning tree into the 2-dimensional space for illustrating genomic distance between populations. **c)** Clustering of fir individuals as inferred with ADMIXTURE when assuming $K=3$ and $K=6$. The purple rectangle below graph denotes admixed populations with C and E ancestries; these stands were grouped in M cluster for analyses shown in figures 4 and 5.

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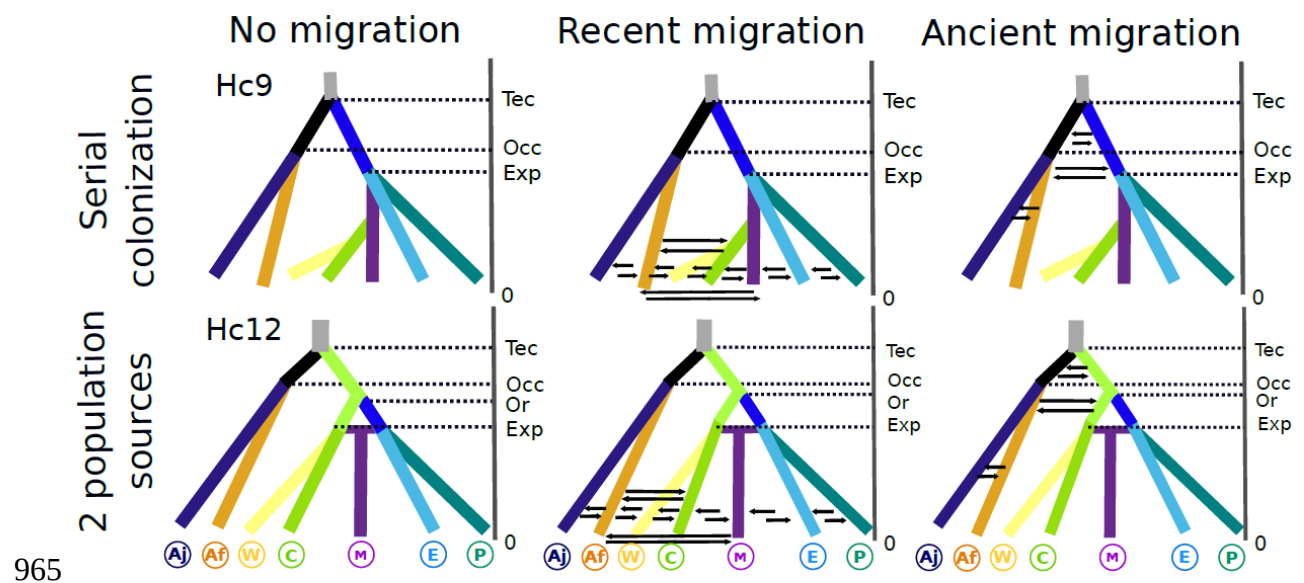
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Figure 4. Demographic models tested for inferring divergence, expansion and gene flow times between fir species/populations in central Mexico. Scenarios considered that populations of *A. religiosa* either colonized its range from a single source (Hc9; top row) or from two sources (Hc12; bottom row). For each pattern, three migration hypotheses were tested: non-migration (left), recent gene flow (center), and ancient gene flow (right). Colors and lineages are the same as in Figure 3.

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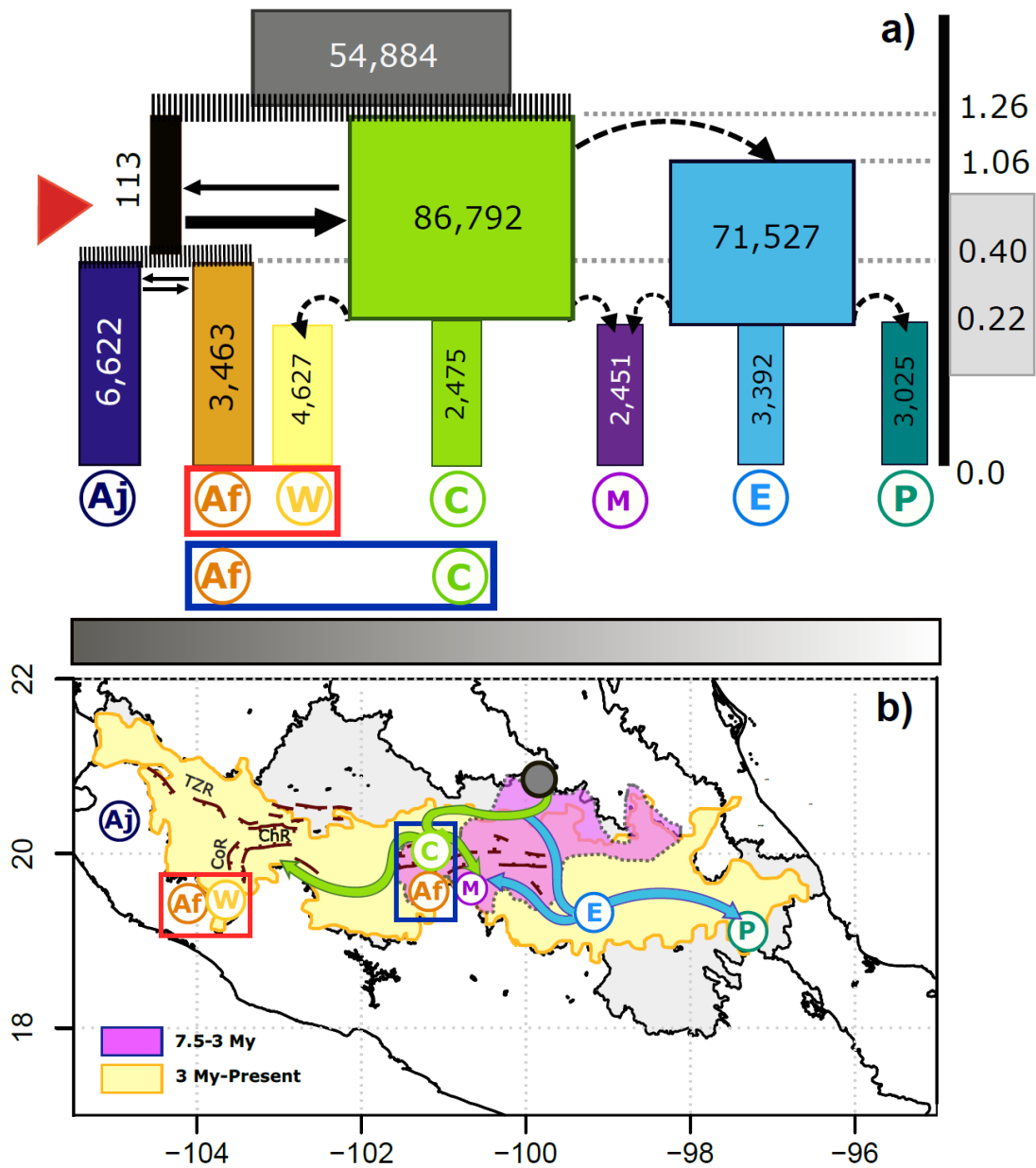
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Figure 5. Graphic representation of the best-fitted divergence and demographic scenario for firs in central Mexico. **a)** Divergence (barcoded lines) and colonization (dotted rounded arrows) times (see time-scale in My on the right; mid-Pleistocene in gray), effective population sizes (N_e s; proportional to rectangle size) and gene flow rate between lineages (proportional to continuous horizontal lines weight). Red triangle shows the proposed time for reproductive isolation between *A. religiosa* and *A. flinckii* (see text). Tones of gray in the bottom horizontal bar correspond to a more fragmented volcanic landscape (dark), and a more continuous landscape (light). **b)** Geographic illustration of events depicted in **a)**, including the putative location of source populations (circles) and dispersal routes (arrows). The two most recent magmatic arcs are represented as purple and yellow polygons (based on Ferrari *et al.*, 2012). The main tectonic valleys (e.g., TZR, Tepic-Zoacalco rift; CoR, Colima rift; Chapala rift) are shown as dark lines (based on Rosas-Elguera *et al.*, 2003). The approximate location of secondary contact zones is indicated by the red (W) and blue (C) rectangles.

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