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Molecular signatures of parallel adaptive divergence causing reproductive isolation and speciation across two genera
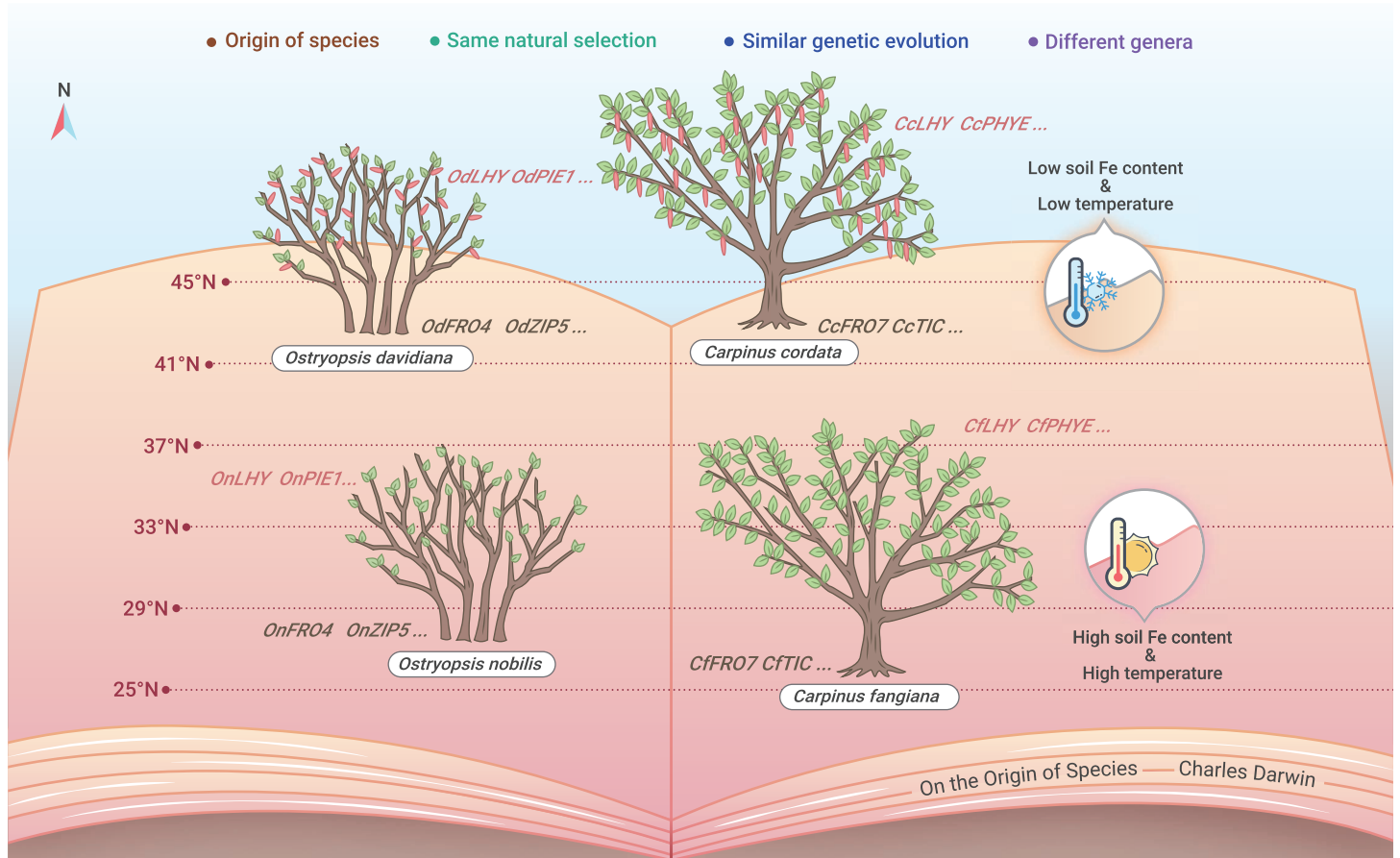
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GRAPHICAL ABSTRACT

PUBLIC SUMMARY

- PERI provides strong evidence for natural selection playing a fundamental role in the origin of species

- PERI is rarely demonstrated for well-established species drawn from different genera

- We detected PERI across two genera (*Carpinus* and *Ostryopsis*) in the family Betulaceae

- PERI can occur in different genera at different timescales and involve molecular signatures at similar pathways

Molecular signatures of parallel adaptive divergence causing reproductive isolation and speciation across two genera

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Parallel evolution of reproductive isolation (PERI) provides strong evidence for natural selection playing a fundamental role in the origin of species. However, PERI has been rarely demonstrated for well established species drawn from different genera. In particular, parallel molecular signatures for the same genes in response to similar habitat divergence in such different lineages is lacking. Here, based on whole-genome sequencing data, we first explore the speciation process in two sister species of *Carpinus* (Betulaceae) in response to divergence for temperature and soil-iron concentration in habitats they occupy in northern and southwestern China, respectively. We then determine whether parallel molecular mutations occur during speciation in this pair of species and also in another sister-species pair of the related genus, *Ostryopsis*, which occupy similarly divergent habitats in China. We show that gene flow occurred during the origin of both pairs of sister species since approximately 9.8 or approximately 2 million years ago, implying strong natural selection during divergence. Also, in both species pairs we detected concurrent positive selection in a gene (*LHY*) for flowering time and in two paralogous genes (*FRO4* and *FRO7*) of a gene family known to be important for iron tolerance. These changes were in addition to changes in other major genes related to these two traits. The different alleles of these particular candidate genes possessed by the sister species of *Carpinus* were functionally tested and indicated likely to alter flowering time and iron tolerance as previously demonstrated in the pair of *Ostryopsis* sister species. Allelic changes in these genes may have effectively resulted in high levels of prezygotic reproductive isolation to evolve between sister species of each pair. Our results show that PERI can occur in different genera at different timescales and involve similar signatures of molecular evolution at genes or paralogues of the same gene family, causing reproductive isolation as a consequence of adaptation to similarly divergent habitats.

INTRODUCTION

Natural selection was assumed by Darwin (1859) to play a critical role in the origin of species.[1] Since then, it has been proposed that natural selection via adaptation and genetic drift by chance can fix alleles at different loci in geographically isolated populations resulting in incompatibility interactions between such loci and the evolution of postzygotic reproductive isolation (RI) leading to allopatric speciation.[2,3] In addition, natural selection can favor the evolution of prezygotic RI barriers during divergence, with these often evolving before postzygotic RI, especially under conditions of continuous gene flow.[4,5] Parallel evolution of RI (PERI), in which RI arises repeatedly as a consequence of adaptation to similarly divergent habitats, provides convincing evidence for natural selection in the generation of new species.[5–7] This is especially true when the evolution of similar molecular mechanisms and convergent mutations underlie the evolution of RI between diverging lineages, because genetic drift is unlikely to generate such concerted evolution in response to similar habitat differences.[4,5,8] To date, tests of PERI have focused on examples of "parallel speciation" involving repeated ecological adaptations and the formation of RI in separate but closely related populations of a single species or between incipient species in complexes at early stages of divergence.[9–12] In such studies, it is necessary to distinguish between the same adaptive mutations occurring independently in different populations and incipient species and, alternatively, that pre-existing adaptive alleles are pre-

sent in them as a result of introgression and/or lineage sorting.[13,14] Here, in contrast with most "parallel speciation" studies, we aimed to examine at the molecular level whether PERI has occurred between well established sister species across different genera in response to similar habitat divergence by exploring allelic changes for genes and members of gene families involved in local adaptation and RI.

Northern and southwestern China are characterized by distinctive environmental differences in average temperature (low vs. high) and soil iron content (low vs. high), respectively.[15,16] Sister plant species distributed in northern and southwestern China, respectively, occur in numerous plant genera and a previous study of two such sister species of *Ostryopsis* (Betulaceae), namely, *Ostryopsis nobilis* and *Ostryopsis davidiana* (Figure 1), showed they were divergent for both iron tolerance and flowering time, which, in turn, caused them to be strongly reproductively isolated from each other owing to habitat divergence.[14] Population genomic studies and functional tests further demonstrated that allelic differences for several major genes (including *LATE ELONGATED HYPOCOTYL* [*LHY*], *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1* [*PIE1*], and *FLOWERING LOCUS PA* [*FPA*]) determined RI owing to flowering time differences, while those for another set of genes (including *ZINC TRANSPORTER 5 PRECURSOR* [*ZIP5*], *FERRIC REDUCTION OXIDASE 4* [*FRO4*], and *HEAVY METAL ASSOCIATED PROTEIN 6* [*HMP06*]) contributed to RI through differences in iron tolerance. The formation of these two *Ostryopsis* species since approximately 9.8 million years ago (mya) was shown to have occurred despite gene flow. It was also demonstrated that hybridization between them led to the origin of a homoploid hybrid species (*Ostryopsis intermedia*), which was reproductively isolated from both parent species owing to the inheritance of alternate alleles of the above parental RI genes.[14]

Another pair of sister species in the related genus *Carpinus* (Betulaceae), namely, *Carpinus cordata* and *Carpinus fangiana*, show similar differences in geographical distribution to the two sister species of *Ostryopsis*, occurring in northern and southwestern China, respectively (Figure 1). These two species, together with *Carpinus japonica*, comprise sect. *Distegocarpus* of *Carpinus*.[17] The species are morphologically distinct with female inflorescences of *C. fangiana* longer than those of *C. cordata*, while the leaves of *C. cordata* are lanceolate or elliptic-lanceolate with 24–34 lateral veins on each side of the midvein, whereas they are ovate to ovate-oblong with only 15–25 lateral veins in *C. fangiana* (Figures 2A and S1).[17] Populations of the extremely endangered *C. fangiana* are small in size and scattered in the temperate forests of southwestern China.[17] In contrast, *C. cordata* is widespread in the temperate forests of northern China and Japan (Figure 1). The two *Carpinus* species show similar abiotic niche differences to the two sister *Ostryopsis* species with regard to both annual average temperature (Figures 1A and 1B) and soil iron content (Figures 1C and 1D), with southwestern *C. fangiana* flowering later and occurring on soils with high iron concentrations compared with northern *C. cordata*. Here, we determined (1) whether the two *Carpinus* species also diverged with gene flow, and (2) if divergence involved similar allelic changes across some of the genes and gene families shown to be important in flowering time and soil-iron tolerance divergence in *Ostryopsis*.[14] Since *Ostryopsis* and *Carpinus* were established in the family Betulaceae by Prantl (1894),[18] they have continued to be acknowledged as distinct genera exhibiting notable morphological differences, especially in floral and fruit traits,[17,19,20] as well as large structural differences in their genomes.[21] The two
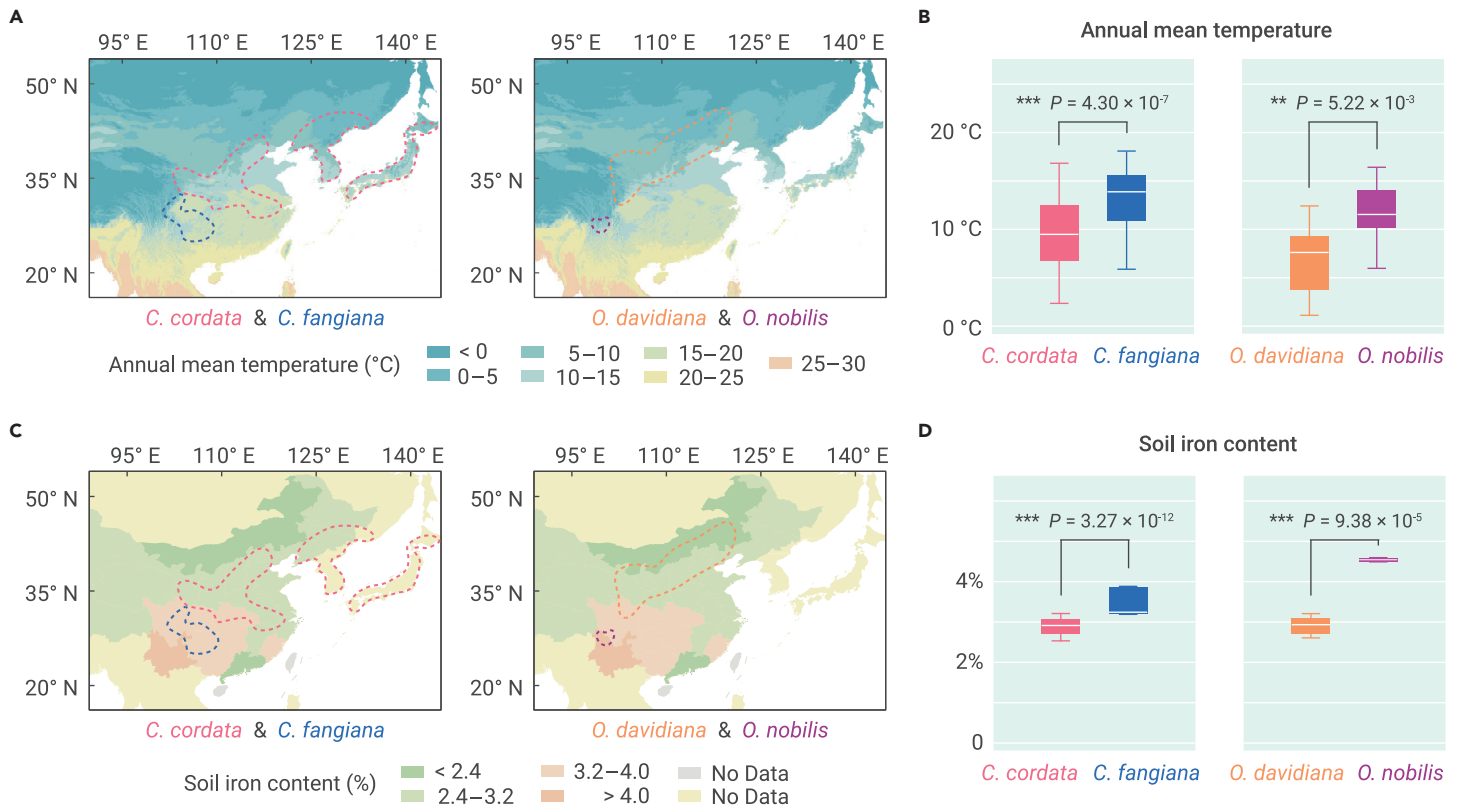
Figure 1. Geographic distributions of two pairs of sister species of *Carpinus* and *Ostryopsis* in eastern Asia (A and B) Annual mean temperature of the geographic distributions of each species. (C and D) Soil iron content of the geographic distributions of each species.

Background of maps show the distribution of (A) annual mean temperature, and (C) soil iron concentrations in the region. Note: boxplots indicate the median, while box edges denote the 25th and 75th percentiles, and whiskers denote the maximum and minimum data points within 1.5× the interquartile range outside the box edges. n > 150 for *C. cordata*, n > 35 for *C. fangiana*, n = 27 for *O. davidiana*, n = 7 for *O. nobilis*. Statistical significance was determined by a two-tailed Student *t* test. Significant differences were indicated with asterisks (**$P < 0.01$, ***$P < 0.001$).

genera initially diverged approximately 25 mya[15,22] and three or more distinct species have evolved within each genus.[17]

RESULTS
Nuclear-plastid genomic discordance of divergence between two *Carpinus* species

We performed whole-genome sequencing (an average of approximately 23 × for each sample) on a total of 69 individuals of *C. cordata*, *C. fangiana*, *C. japonica*, and three individuals of *O. nobilis*, and generated 4,562,758 high-quality biallelic SNPs for them (Figure 2A, Tables S1 and S2). To examine population genetic structure, we performed an ADMIXTURE analysis on *Carpinus* individuals and phylogenetic analysis on all individuals (with *O. nobilis* as outgroup) using the nuclear genome SNP variation. With *K* = 3 in the ADMIXTURE analysis, *C. cordata*, *C. fangiana*, and *C. japonica* formed three distinct groups (Figure 2B). These groupings were consistent with the nuclear genomic tree topology, where each species formed a monophyletic clade, and *C. cordata* and *C. fangiana* were sister to each other (Figure 2C). Within each species, individuals in the phylogenetic tree clustered according to geographic distribution, indicating the occurrence of population genetic structure (Figures 2A and 2C). Individuals of *C. cordata* formed two clades: one containing most individuals sampled from northern China and the other comprising all individuals from Japan plus three individuals from northern China (Figures 2A and 2C). For *C. fangiana*, individuals from Guizhou (GZ) and Sichuan (SC) in southwestern China formed two monophyletic clades (Figures 2A and 2C). Our nuclear genome analyses therefore showed that *C. cordata* and *C. fangiana* are sister species that are easily delimited genomically, as they are morphologically (Figures 2A–2C), and further they had diverged from each other approximately 2 mya (Figure S1).

An analysis of population plastome data identified four lineages (clades A–D, Figure 2D) that were discordant from those identified by nuclear genome analysis. Based on secondary calibration (see more details in online supplemental materials and methods), the two relatively young clades (clades C and D) diverged approximately 2.95 mya, i.e., before the divergence of *C. cordata* and

C. fangiana (approximately 2 mya) based on nuclear genomic variation (Figures 2D and S1). However, the plastomes recovered from the two species were nested within both clades C and D and, therefore, did not represent monophyletic subclades. Moreover, they diverged approximately 2 mya (2.17–2.46 mya), corresponding with the divergence time of the two species estimated from nuclear genome analysis (Figures 2D and S1). In addition, we recovered two clades for the widespread *C. cordata* (clades A and B) that had diverged earlier (approximately 5.10 mya and approximately 4.47 mya). Both of these clades were distributed in Japan or northeastern China, far from the distribution of *C. fangiana* and the other two clades comprising *C. cordata* individuals (clades C and D) (Figures 2A and 2D). During speciation, such nuclear-plastid phylogenetic discordance may have arisen owing to gene flow (hybridization between diverging species without complete RI) and/or incomplete lineage sorting,[23–27] although other factors, including hidden paralogy and horizontal gene transfer, cannot be excluded.[28]

Speciation of *C. cordata* and *C. fangiana* with gene flow

To investigate the speciation process resulting in the origins of *C. cordata* and *C. fangiana*, we first examined historical changes in effective population size (N_e) (Figure 3A). Since their initial divergence (approximately 2 mya), the N_e of both species declined but in different ways, reflecting their unique evolutionary histories. For *C. fangiana*, the N_e of SC populations (Cfa-SC) declined much more slowly than the N_e of GZ populations (Cfa-GZ) until the Mindel glacial stage (0.30–0.45 mya), during which a rapid decline of N_e occurred in SC populations. It is possible that the declines of N_e in both species were triggered by past climatic oscillations that, according to our ecological niche modeling, also caused changes in the species distributions, leading to periods of overlap and likely gene flow (Figure S2, Tables S3 and S4).

An analysis of haplotype sharing showed that individuals of the same species shared more and longer identity-by-descent blocks than those from different species (Figures 3B and S3B). Moreover, within each species, individuals from the same geographical region shared more haplotypes than those from different
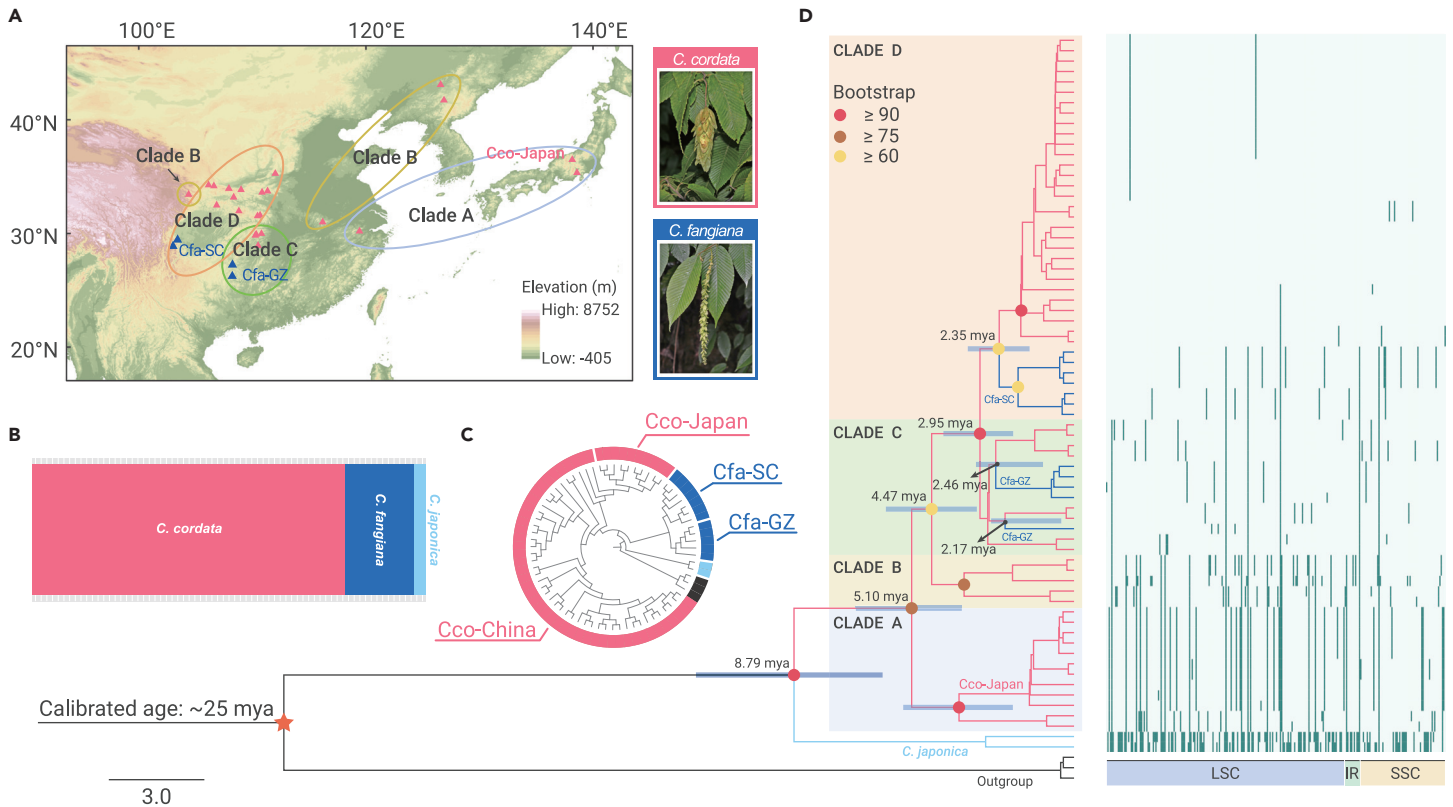
Figure 2. Plastid-nuclear discordance in genomic divergence (A) Distributions of individuals sampled for sequencing and the phenotypes of *Carpinus cordata* and *C. fangiana*. Clades A–D refer to different plastome clades in the phylogenetic topology (shown in D). (B and C) Population structure (B) and phylogenetic topology (C) based on nuclear genomes of all individuals sampled. (D) Phylogenetic topology, divergence time, and variant sites of plastomes. Bars on the nodes indicated 95% confidence intervals of estimated times. *Ostryopsis nobilis* were used as the outgroup. LSC, SSC, and IR denote the large single copy region, small single copy region, and one inverted repeat region of plastomes, respectively.

ones (Figure 3B). Nevertheless, some haplotype sharing was detected between *C. cordata* and *C. fangiana* (Figures 3B and S3B), which might be attributed to gene flow and/or incomplete lineage sorting. Strong evidence for gene flow being a cause was indicated by significant Z values obtained in ABBA-BABA tests (Figures 3C and S3C).

Further evidence for gene flow having occurred during the divergence of the two *Carpinus* species was obtained from coalescent simulations that examined their demographic history. The best supported model (model 5) suggests that continuous gene flow occurred since the initial divergence of the two species with gene flow occurring mainly from the widespread *C. cordata* in northern China to the narrowly distributed *C. fangiana* in southwestern China (Figure 3D and Table S5).

To explore the genomic effect of gene flow during speciation, we examined the correlations between different genomic parameters (Figures 3E, S4 and S5). This showed that relationships between recombination rates and d_{XY} were opposite in the two species, with genomic regions exhibiting high recombination rates in *C. fangiana* showing a lower level of d_{XY}. This suggests that *C. fangiana*, with much lower population sizes, was more sensitive to interspecific gene flow. Moreover, between the two species, those genomic regions with high d_{XY} values always exhibited highest levels of F_{ST}, indicating that species-specific haplotypes, including those with genomic islands of ancient origin, experienced strong divergent sorting and divergence hitchhiking during natural selection.

Positively selected genes and PERI in response to environmental selection

Speciation with gene flow leads to the fixation of adaptive alleles that can directly or indirectly cause RI.[13,29] To isolate such alleles, we identified positively selected genes (PSGs) involved in adaptive divergence and RI between the sister species of *Carpinus*, following an approach outlined by Ma et al.[13,14] (2018) and Wang et al.[13,14] (2021). Among a total of 569 PSGs detected in *C. cordata* or *C. fangiana* (Figure 4 and Table S6), multiple genes had homologs in *Arabidopsis* with functions related to iron tolerance or flowering time (Tables S6–S8), of which

five had previously been examined in the pair of *Ostryopsis* species.[14] For these five genes, two had homologs involved in iron tolerance (*FERRIC REDUCTION OX-IDASE 7* [*FRO7*] and *TIME FOR COFFEE* [*TIC*]),[30,31] while the other three (*LHY*, *PHYTOCHROME E* [*PHYE*], and *CRYPTOCHROME 1* [*CRY1*]) had homologs affecting flowering time[32–34] (Figures 4, S6–S10 and Tables S6–S8).

Functional tests previously showed that three PSGs in *Ostryopsis* were involved in soil iron tolerance (*FRO4*, *ZIP5*, and *HMP06*) and another three affected flowering time (*LHY*, *PIE1*, and *FPA*) (Figure 4D).[14] For PSGs identified in *Carpinus* with homologs or orthologs with those in *Ostryopsis*, we conducted functional tests on *FRO7* and *LHY* alleles that differentiated *C. cordata* and *C. fangiana*. We found the activity of an enzyme encoded by an allele of *FRO7* common in *C. fangiana* (*CfFRO7*) was lower than that encoded by an allele common in *C. cordata* (*CcFRO7*; Figure 5A). A similar result was previously found from comparisons of the activity of enzymes produced by *FRO4* alleles in *Ostryopsis* species with lower activity expressed by the enzyme produced by an allele fixed in *O. nobilis* (*OnFRO4*), which grows on iron-rich soil in southwestern China relative to that of the allele fixed in *O. davidiana* (*OdFRO4*) associated with iron-poor soils in northern China.[14] Lower enzyme activity results in a decrease in iron uptake and assimilation from soils of high iron content.[14]

As a transcriptional repressor, *LHY* could negatively regulate *TIMING OF CAB EXPRESSION 1* (*TOC1*) by binding to the EE (evening element) motif in its promoter region.[32] To test the function of *LHY* alleles in the two *Carpinus* species, we constructed an artificial promoter consisting of three repeats of the EE motif followed by a 35S minimal promoter (Figure 5B). We found that the *LHY* allele from *C. cordata* (*CcLHY*) showed lower repression ability to this artificial promoter, and therefore higher *LUC* expression, relative to the *C. fangiana* allele (*CfLHY*; Figure 5C). Therefore, *C. cordata* may be expected to flower earlier than *C. fangiana*, given that *LHY* negatively regulates flowering times in plants.[35,36] Again, this finding is consistent with those obtained from transgenic tests of the function of two *LHY* alleles distinguishing the two *Ostryopsis* sister species (*OdLHY* and *OnLHY*).[14] A common garden study of those two *Ostryopsis* species further showed that the low-latitude one from southwestern China flowered later than the high-latitude one from northern China (Figures 1A and 1B).[14]
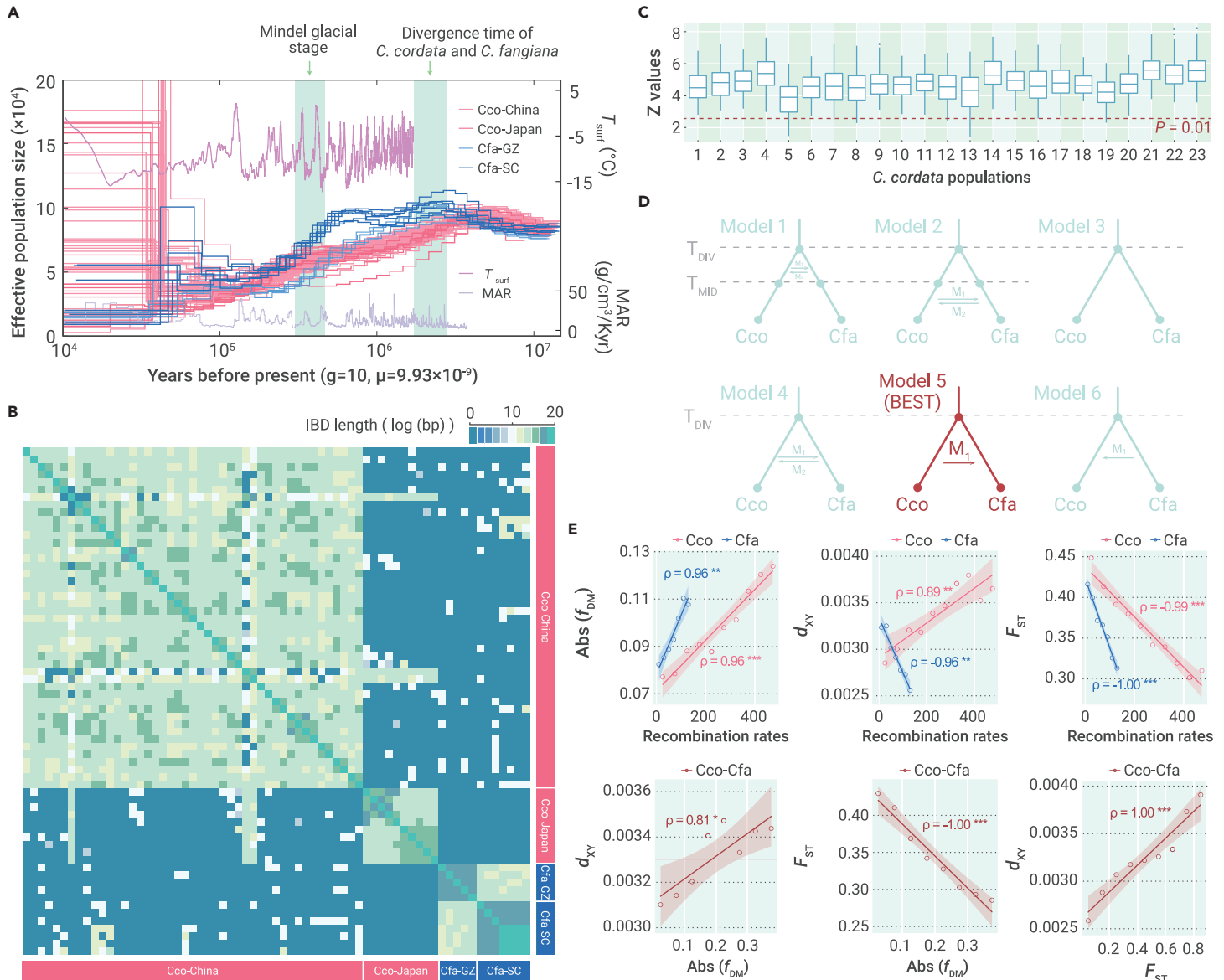
Figure 3. Evolutionary history of *Carpinus cordata* and *C. fangiana* (A) Historical changes in N_e inferred by the pairwise sequentially Markovian coalescent (PSMC) model. (B) The identity-by-descent (IBD) haplotypes shared between each pair of individuals sampled for sequencing. Cco and Cfa refer to *C. cordata* and *C. fangiana*, respectively. Cfa-GZ and Cfa-SC refer to *C. fangiana* populations in Guizhou Province (China) and Sichuan and Guizhou Province (China), respectively. (C) Summary of ABBA-BABA statistics. *C. fangiana* from SC, *C. fangiana* from GZ, *C. cordata*, and *O. nobilis* were set as P_1, P_2, P_3, and O, respectively. The numbers in the x axis and y axis were the population ID of *C. cordata* and the Z values for each test, respectively. The boxes and whiskers represent the maximum and minimum data points within 1.5× the interquartile range outside the box edges. The dots indicate outlier values. (D) Schematic of alternative demographic scenarios modeled in fastsimcoal2. Model 5 indicates the best-supported model in simulations. (E) The correlation for population genetics parameters. Recombination rates were measured for each species. F_{ST} and d_{XY} values were calculated between the two species. When calculating f_{dM} values, *C. fangiana* from SC, *C. fangiana* from GZ, *C. cordata*, and *O. nobilis* were used as P_1, P_2, P_3, and O, respectively. The average values (shown in the y axis) for every interval (shown in the x axis) were calculated and used in a Spearman correlation test. The 95% confidence intervals are shadowed. Significant differences are indicated with asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). For (A–E), see more details in online Methods.

DISCUSSION

Past reports of PERI in both animals and plants have focused on different populations of a single species or closely related species within a species complex.[5,11,37–43] To demonstrate PERI, it is necessary to distinguish multiple independent origins of RI caused by the same type of strong divergent natural selection, from RI owing to introgression and/or sorting of ancestral adaptive polymorphisms.[44] If introgression and sorting of ancestral polymorphisms can be ruled out, as should be the case when dealing with PERI across different reproductively isolated genera, it becomes easier to prove that natural selection is the driver of replicated divergence and RI. In this study, using whole-genome resequencing and functional tests of alleles that contribute to RI, we obtained a molecular signature of adaptive divergence causing RI and speciation in the genus *Carpinus* very similar to that reported previously in the related genus *Ostryopsis*.[14] Although transgenic tests on diverged alleles in the two *Carpinus* species using model plant mutants are still required, so as to determine their effects on phenotype, the available evidence suggests that PERI driven by natural selection occurred independently across both genera, but at different timescales.

Strong natural selection can result in the divergence of populations (and ultimately speciation) in the presence of gene flow and, therefore, in the absence of complete geographic isolation.[13,29,45,46] Our population genomics study indicated that gene flow occurred during the origin of the two sister *Carpinus* species approximately 2 mya based on the following evidence. First, there was phylogenetic discordance between the nuclear and plastid (chloroplast) genomes analyzed in the two species. Whereas the two morphologically divergent species were readily distinguished by their divergent nuclear genomes (Figures 2A–2C), they could not be distinguished by an analysis of plastome variation, which showed each species did not comprise a monophyletic subclade (Figure 2D). In one of the species (*C. cordata*), two ancient plastome clades (clades A and B) were present, in addition to two more recently originated plastome clades (clades C and D) found in both species. According to our analysis, the two ancient plastome clades diverged (approximately 5.10 and approximately 4.47 mya,
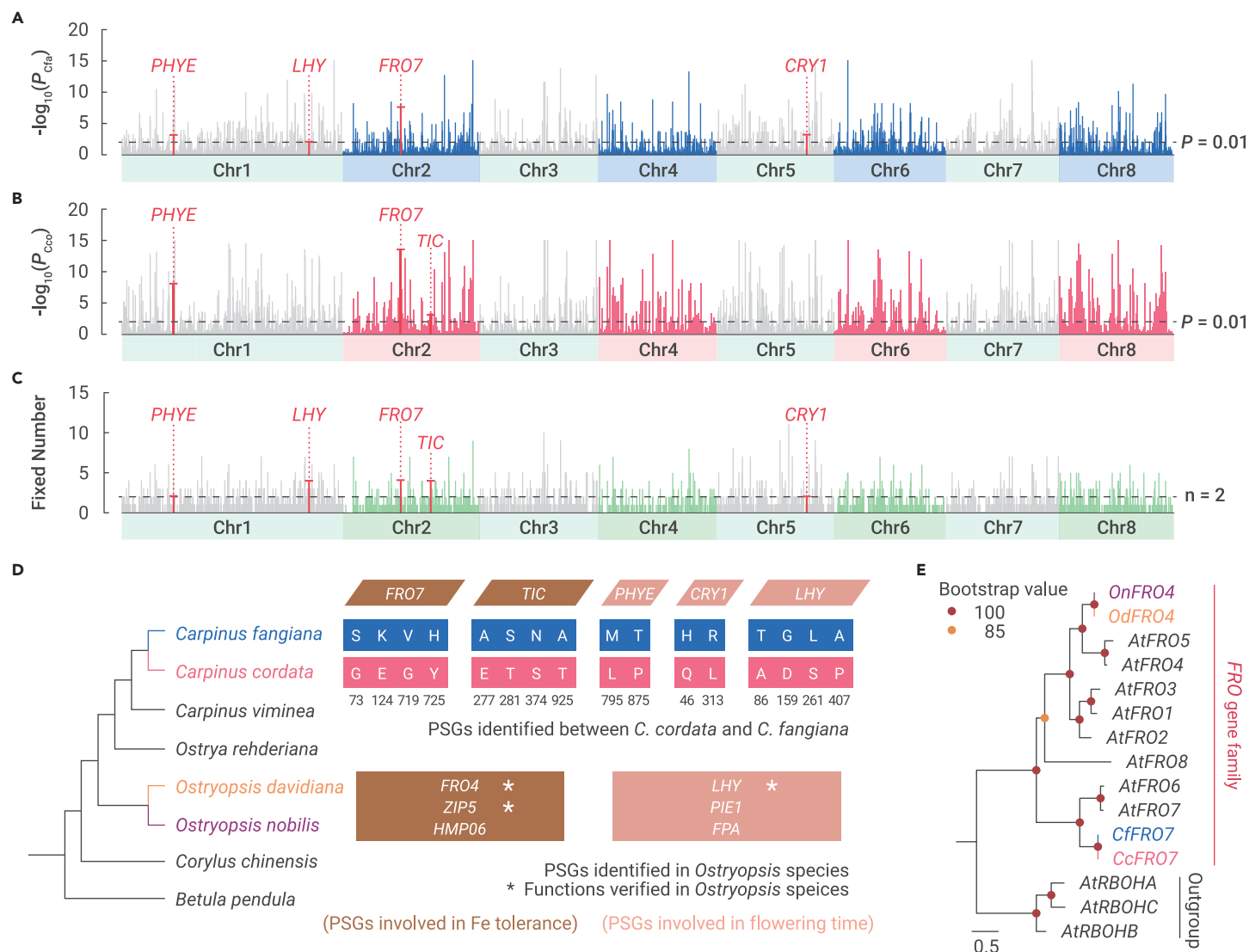
Figure 4. PSGs and PERI (A−C) The likely PSGs identified in *C. fangiana* and *C. cordata*. The bars indicate the *P* values of HKA test in *C. fangiana* (A), *C. cordata* (B), and the number of fixed non-synonymous sites between the two species (C), respectively. The red bars and gene names indicate the identified PSGs involved in iron tolerance and flowering time. (D) Phylogenetic relationships and the identified PSGs involved in iron tolerance and flowering time between the two *Carpinus* species and the two *Ostryopsis* species. The fixed changes in amino acids between the two *Carpinus* species are shown. (E) Maximum likelihood tree based on *FRO* protein sequences in *Carpinus*, *Ostryopsis*, and *Arabidopsis*. Three genes of *Arabidopsis RBOH* gene family served as outgroup.

respectively), that is, before the nuclear genomes (approximately 2 mya) that distinguished the two species (Figures 2D and S1). This indicates that geographic isolation, which likely led to divergence of the ancient plastomes, did not also lead to the origin of strong RI between plants possessing different plastomes. Nuclear-plastid discordance in phylogenomic analyses often suggests strong gene flow during the speciation process, although incomplete lineage sorting cannot be completely excluded.[23−27] A second line of evidence suggesting that speciation with gene flow resulted in the origin of the two *Carpinus* species was the finding that many nuclear haplotypes were shared between them (Figures 3B and S3B) and that ABBA-BABA tests indicated this was due to gene flow rather than incomplete lineage sorting (Figures 3C and S3C). Further support was also obtained from coalescent simulations of the historical demography of the two species. These indicated that continuous gene flow had occurred between the species since their initial divergence (Figure 3D and Table S5). Given that gene flow had previously been shown to occur between the pair of *Ostryopsis* species during their divergence since approximately 9.8 mya,[14] we may conclude that strong natural selection drove divergence in both pairs of species.

Evidence that molecular changes underlying adaptive divergence and causing RI between the two *Carpinus* species are similar to that previously reported for the pair of *Ostryopsis* species emerged from an analysis of PSGs (Figures 4 and 5). First, we found some PSGs likely to be involved in iron uptake in *Carpinus*, similar to those previously detected in *Ostryopsis* (Figure 4D). Among these were two

PSGs (one present in *Carpinus*—*FRO7*, and the other in *Ostryopsis*—*FRO4*), which are paralogs of the same gene family exhibiting similar functions (Figures 4D and 4E). Functional tests showed that an allele of *FRO7*, which occurred at high frequency in *C. fangiana* (*CfFRO7*) distributed on high-iron soils, encoded a low activity enzyme as previously reported for the enzyme encoded by an allele of *FRO4* in iron-tolerant *O. nobilis* (*OnFRO4*). In contrast, iron-sensitive *C. cordata* and *O. davidiana*, which both occur on soils having much lower iron contents, possessed alleles of *FRO7* (*CcFRO7*) and *FRO4* (*OdFRO4*), respectively, that encoded enzymes exhibiting significantly higher activities (Figure 5A). The previous study of the pair of *Ostryopsis* species showed that allelic divergence at *FRO4* (*OdFRO4* and *OnFRO4*), and possibly other major genes involved in iron tolerance, enabled the two species to grow on soils of markedly different iron content, thus contributing to their RI through a high level of habitat divergence.[14] Further experiments are required to confirm that allelic divergence at *FRO7* (*CcFRO7* and *CfFRO7*, and possibly other major genes involved in iron tolerance) similarly enable the two species of *Carpinus* to grow on soils that differ in iron content, thereby contributing to their RI. However, this is predicted from the different activities shown by the enzymes they encode, which parallel those exhibited by the different alleles of *FRO4* in *Ostryopsis* (*OdFRO4* and *OnFRO4*).

In addition to detecting allelic differences of adaptive importance for *FRO* genes, allelic differences were detected at PSGs related to flowering time in both pairs of species (Figure 4D). This was especially apparent for the single
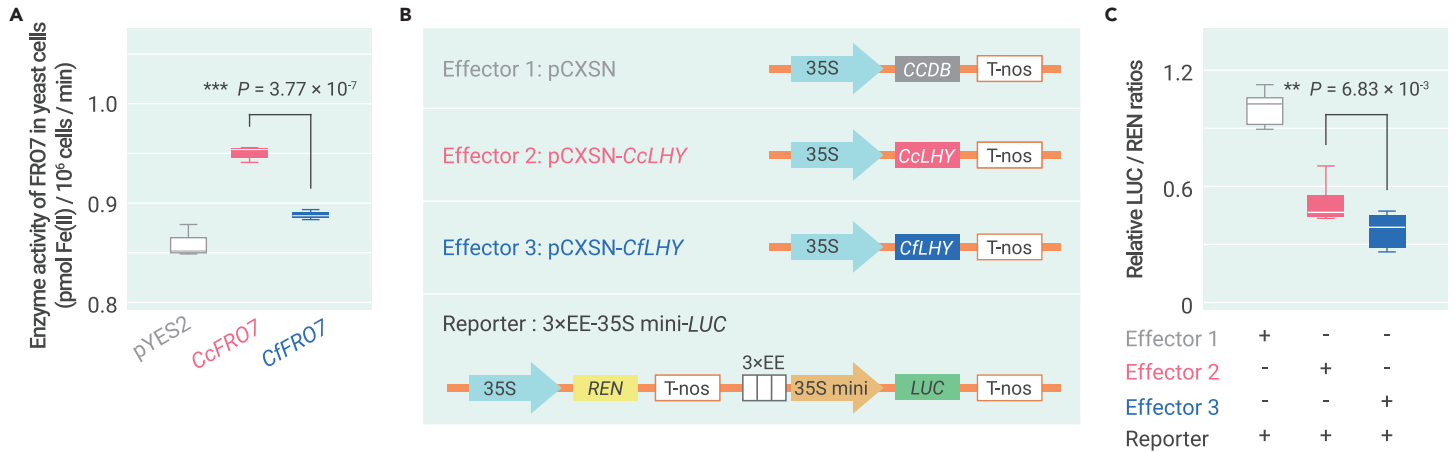
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Figure 5. Functional divergence of *FRO7* and *LHY* alleles in *Carpinus* (A) Enzyme activity of the commonest *FRO7* allele from each species. The y axis denotes the Fe(III) reductase activity. (B and C) Dual-luciferase assay of the commonest *LHY* allele from each species, indicating their functional differentiation of repression ability to the binding element (C). Effector and reporter structures by dual-luciferase assay are shown in (B).
Note: boxplots show the median, box edges denote the 25th and 75th percentiles, and whiskers denote the maximum and minimum data points within 1.5× the interquartile range outside the box edges. (A) n = 5; (C) n = 9. Statistical significance was determined by a two-tailed Student *t* test. Significant differences were indicated with asterisks (**$P < 0.01$, ***$P < 0.001$).

gene *LHY*. *LHY* acts as a transcriptional repressor[32] and a loss-of-function *LHY* mutant has been shown to enhance expression of the flowering time gene *FT*, leading to early flowering in *Arabidopsis*.[35,36,47] Our results indicate that the *LHY* allele, which commonly occurs in *C. cordata* (*CcLHY*), exhibits weaker transcriptional repression ability relative to the *LHY* allele present in *C. fangiana* (*CfLHY*; Figure 5C). Although confirmatory transgenic tests remain to be conducted, we speculate that the *LHY* allele commonly found in northern *C. cordata* (*CcLHY*) causes it to flower earlier than its sister species, *C. fangiana*, which is restricted to southwestern China. In support, the previous study of the sister pair of *Ostryopsis* species, which involved both common garden experiments and transgenic tests of the effects of *Ostryopsis LHY* alleles (*OdLHY* and *OnLHY*) on flowering, produced strong evidence that the *LHY* allele present in the northern species (*OdLHY*) promoted early flowering.[14]

CONCLUSIONS

Our results indicate that natural selection in response to similarly divergent environmental conditions may have resulted in adaptive divergence causing RI and speciation in two different plant genera, *Carpinus* and *Ostryopsis* (Figures 6 and S11). Molecular signatures of this PERI across these two genera were obtained for alleles at genes influencing iron tolerance and flowering time variation. The selective advantage of different alleles of these genes in different environments was likely involved in the origin of sister species in both genera, which differ in ability to grow on soils of low and high iron content in northern and southwestern China, respectively. As a result of habitat divergence and inherited differences in flower-

ing time, the two species of each pair are strongly reproductively isolated from each other. This finding therefore provides evidence for PERI likely occurring at the genus level and highlights the importance of natural selection in speciation.

The evolution of prezygotic RI mechanisms, which we have demonstrated to have occurred in both *Carpinus* and *Ostryopsis* species pairs at different timescales, is regarded as a potent force in speciation[2,5] and, in plants, often contributes more to total RI than do postzygotic barriers.[7] Prezygotic RI barriers are expected to evolve commonly as an outcome of adaptive divergence to different environments, involving, for example, changes in habitat and/or pollinators. In contrast, postzygotic RI arises as a by-product of such genetic divergence or as a consequence of genetic drift under conditions of geographic isolation.[3,48–50] Darwin (1859) assumed that natural selection is likely often to play a critical role in speciation,[1] and our findings highlight the importance of natural selection in speciation through the same or a similar genetic pathway. In addition, we propose that PERI across genera might be detected frequently whenever adaptive divergence occurs in different genera in response to similar environmental differences. It may occur by the formation of single or multiple prezygotic RI barriers, for example, habitat and flowering time isolation, as reported here. Furthermore, in addition to amino acid changes, other forms of genetic divergence, such as in non-coding changes, causing changes of gene expression through allelic variation in regulatory regions, could lead to such PERI. However, it remains to be explored whether these forms of PERI could directly or indirectly lead to parallel postzygotic RI. The results of our study set the stage for a future examination of all of these possibilities.
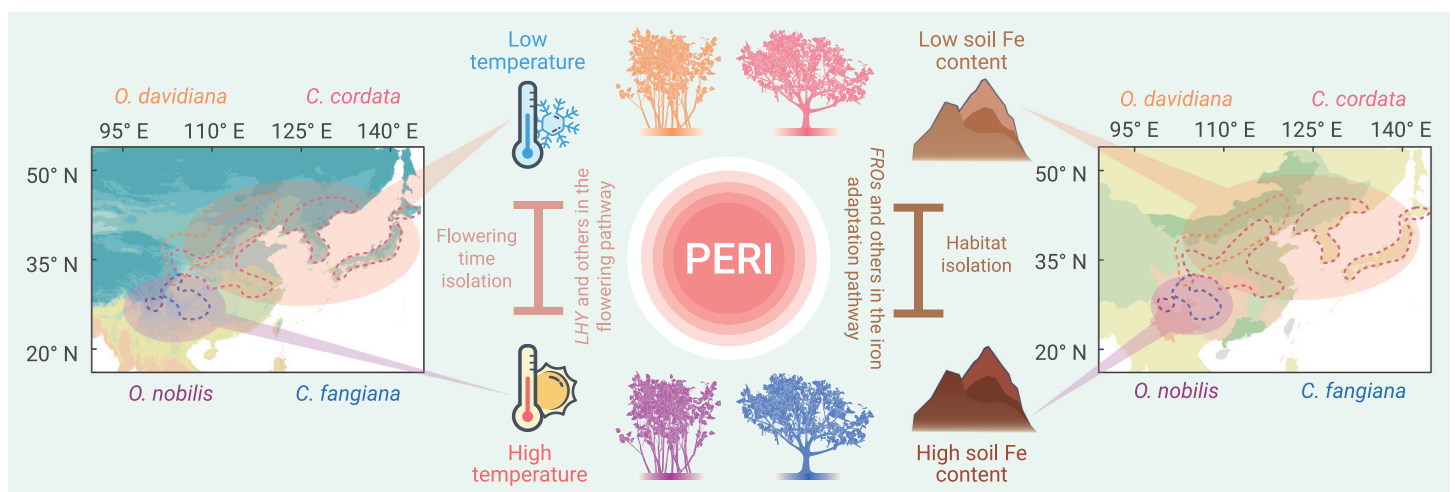

Figure 6. Simplified schematic of PERI in *Carpinus* and *Ostryopsis* in response to habitat differences in average temperature and soil iron content

MATERIALS AND METHODS

See supplemental information for details.

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ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

J. Liu designed and led the project. Z.W. conducted the research. X.M. and G.H. collected the materials. Z.W. and X.Y. prepared DNA for sequencing and analyzed the data. Y.J. and H.B. performed the functional tests of alleles. J. Li, Y.M., D.R., C.Z., and J.W. contributed to data analyses. Z.W. wrote the manuscript. J. Liu and R.J.A. revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xinn.2022.100247.

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