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Research

Sequential hybridization may have facilitated ecological transitions in the Southwestern pinyon pine syngameon

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Summary

- Multispecies interbreeding networks, or syngameons, have been increasingly reported in natural systems. However, the formation, structure, and maintenance of syngameons have received little attention. Through gene flow, syngameons can increase genetic diversity, facilitate the colonization of new environments, and contribute to hybrid speciation.
- In this study, we evaluated the history, patterns, and consequences of hybridization in a pinyon pine syngameon using morphological and genomic data to assess genetic structure, demographic history, and geographic and climatic data to determine niche differentiation.
- We demonstrated that *Pinus edulis*, a dominant species in the Southwestern US and a barometer of climate change, is a core participant in the syngameon, involved in the formation of two drought-adapted hybrid lineages including the parapatric and taxonomically controversial *fallax*-type. We found that species remain morphologically and genetically distinct at range cores, maintaining species boundaries while undergoing extensive gene flow in areas of sympatry at range peripheries.
- Our study shows that sequential hybridization may have caused relatively rapid speciation and facilitated the colonization of different niches, resulting in the rapid formation of two new lineages. Participation in the syngameon may allow adaptive traits to be introgressed across species barriers and provide the changes needed to survive future climate scenarios.

Introduction

Natural hybridization has played an important role in the evolution of plants, birds, fish, fungi, insects, and mammals, including humans (Mallet, 2005; Abbott *et al.*, 2013; Taylor & Larson, 2019). Although most studies on hybridization assess gene flow between two species in sympatry (see Whitney *et al.*, 2010), hybridization can occur among multiple species simultaneously, with well-documented examples in cichlids, oaks, heliconias, sunflowers, corals, eucalypts, and pinyon pines among others (Grant, 1981; Rieseberg, 1991; Schliewen & Klee, 2004; Mallet, 2007; Mallet *et al.*, 2007; Ladner, 2012; Flores-Rentería *et al.*, 2017; Buck *et al.*, 2020). These multispecies interbreeding networks were defined as syngameons almost a century ago (Lotsy, 1925), and here we use the term to mean three or more distinct species connected by gene flow (Buck & Flores-Rentería, 2022). Despite the long recognition of syngameon existence, our understanding of how multiple species can collectively exchange genes, and the resulting evolutionary consequences, is limited (Buck & Flores-Rentería, 2022). It is not understood how participating in a syngameon has affected the evolutionary history of species and how it may affect their future evolutionary trajectories, but some evidence shows syngameons may be responsible for rapid radiations, niche diversification, and even island colonization (Seehausen, 2004; Caujapé-Castells & Bramwell, 2011; Caujapé-Castells *et al.*, 2017; Liu *et al.*, 2017).

One hypothesis proposes that speciation events could be more common in a syngameon due to the increased amount of hybrid pairs (Seehausen, 2004; Liu *et al.*, 2017). If a hybrid speciation event occurred within a syngameon, would reproductive barriers form at all, and if they did, would they remain and exclude the new hybrid species from joining the syngameon or would they quickly dissipate to enable syngameonic participation? While reproductive barriers are not necessarily a requirement for hybrid speciation (Mallet, 2007; Mavárez & Linares, 2008; Cannon, 2021), understanding syngameons can give insight into the processes of speciation under gene flow and in sympatry. It is also not clear how species are able to maintain their identities in the face of extensive gene flow yet simultaneously act as a collective gene pool (Cannon & Petit, 2020). Spatial and temporal patterns of gene flow, as well as its direction and magnitude, constitute the structure of a syngameon (Ladner, 2012; Boecklen, 2017); but it is not known how these patterns affect the stability of the entire hybrid network. For example, intermittent waves of gene flow could facilitate the periodic introduction of genetic variation while providing enough time for traits to sort, allowing lineages to remain distinct (Cannon & Lerdau, 2015). Alternatively, constant interspecific gene flow could supply adaptive traits when needed through a sustained pool of genetic variation (Givnish, 2010; Racimo *et al*., 2015; Dannemann & Racimo, 2018; Menon *et al*., 2021; Storz & Signore, 2021).

Pinyon pines (subsection *Cembroides*) are prone to hybridization due to their lack of genetic isolating mechanisms (Critchfield, 1975), partially overlapping distributions (Mansfield-Jones Jr, 1967; Lanner, 1971; Little, 1999; Cole *et al*., 2013), and long-distance, wind-mediated pollination with overlapping pollen dispersal times (Malusa, 1992; Farjon & Styles, 1997; Williams, 2010). This is further supported by the intermediate morphology observed in sympatry (Lanner, 1974a; des Lauriers & Ikeda, 1986). Therefore, pinyon pines are an excellent system to explore these multispecies hybridization dynamics and outcomes due to the known presence of a syngameon with at least three hybridizing species in the Southwestern US that have varying patterns of hybridization (Buck *et al*., 2020) and unique ecological niches (Cole *et al*., 2008; see Supporting Information Notes S1). Buck *et al.* (2020) was the first conclusive genetic study to demonstrate syngameonic hybridization in the Southwestern pinyon pines, examining gene flow among *Pinus monophylla*, *P. quadrifolia*, and *P. californiarum*. This investigation includes two additional taxa that may also participate in this pinyon pine syngameon: *P. edulis* and the taxonomically controversial *fallax*-type (Lanner, 1971, 1974a,b; Lanner & Hutchison, 1972). *Pinus edulis* and the hypothesized *fallax*-type form a monophyletic group with the other three syngameon participants (Montes *et al*., 2022) and partially overlap in distribution (Fig. 1). Understanding whether these taxa form part of the syngameonic network is critical because *P. edulis* is a dominant species in the Southwestern US and a barometer of climate change (Gitlin *et al*., 2006), experiencing massive rates of mortality after recent drought events (Ogle *et al*., 2000; Mueller *et al*., 2005, 2019). *Pinus edulis* is a foundation species, supporting thousands of other species above and belowground, including its avian and rodent seed dispersers (i.e. the scrub, pinyon, and Steller's jays, Clark's nutcracker, chipmunks, squirrels, deer mice, pinyon mice, Great Basin pocket mice, and Panamint kangaroo rats; Gottfried, 1987; Hollander & Vander Wall, 2004), so its mass mortality has a large effect on the ecosystem it supports (Paulin *et al*., 1999; Gehring *et al*., 2014; Stone *et al*., 2018; Patterson *et al*., 2019). Compared with the other taxa in this syngameon, *P. edulis* inhabits higher elevation areas with monsoon-like precipitation, whereas *fallax*-type inhabits areas with monsoon-like precipitation but drier by 12 mm and hotter by 2–4°C, showing that *P. edulis* and *fallax*-type have different niches (Cole *et al*., 2008). If *P. edulis* does participate in this syngameon, natural hybridization could facilitate the exchange of genes from more arid-adapted taxa into *P. edulis*, potentially allowing it to withstand the changing climate and prevent further die-off. Looking at interspecific gene flow, examining demographic history and establishing species barriers in this complex are therefore crucial to understanding the structure of a syngameon and the future of these species and the species they support. Also, syngameon networks might play a role in maintaining biodiversity in the face of climate change (Cannon & Petit, 2020).

While hybridization between *fallax*-type and *P. edulis* has been proposed (Lanner & Phillips, 1992; LaHood, 1995), no genetic studies have found evidence of admixture between the two taxa (Duran *et al*., 2012; Montes *et al*., 2019) and Montes *et al.* (2022) found no genetic differentiation between them, implying that the two are one taxon. *Fallax*-type typically has one needle per fascicle with two resin canals (Little, 1968; Cole *et al*., 2008), whereas *P. edulis* has two needles and two resin canals. *Fallax*-type's mixture of traits might suggest a hybrid origin between the single-needled *P. monophylla* and the two-resin-canaled *P. edulis* (Businský, 2008). However, while intermediate morphology can indicate hybridization (Mavárez *et al*., 2006; Rieseberg *et al*., 2007), individuals with hybrid ancestry are often indistinguishable from their parental species after a few generations of backcrossing (Rieseberg *et al*., 1993; López-Caamal & Tovar-Sánchez, 2014; Buck *et al*., 2020), so it is unclear why admixed individuals would have this consistent and distinguishable *fallax*-type morphology (Bailey, 1987; Cole *et al*., 2008). Further, while *fallax*-type itself overlaps in distribution with *P. edulis*, it rarely occurs in the areas where *P. edulis* and *P. monophylla* overlap. The unique niche occupied by *fallax*-type (Cole *et al*., 2008), along with its distinct morphological traits, suggests that it may either be a valid taxon or an incipient species, regardless of its hybrid origins. However, if *fallax*-type does have a hybrid origin and is also its own species, it would be a rare case of hybrid speciation (Rieseberg, 1997; Goulet *et al*., 2017). Alternatively, *fallax*-type might currently be undergoing speciation, providing an extraordinary glimpse into ongoing incipient speciation processes, especially in the face of extensive syngameonic gene flow.

In this study, we generated morphological and genomic data from 46 populations of the five taxa hypothesized to participate in the pinyon syngameon present in Southwestern North America (Fig. 1). We used these data to: (1) determine whether *P. edulis* and *fallax*-type participate in the known pinyon pine syngameon; (2) elucidate whether any syngameon members (i.e. *fallax*-type) are a result of hybrid speciation; and (3) assess the structure of the syngameon and explore its evolutionary history.

Materials and Methods

Sampling

A total of 963 branch samples were taken from 47 locations across the Southwestern US and Baja California (Table S1), randomly sampling 10 trees per putative species per site, at least 30 m apart from each other. It is important to note that while morphology and geography (per Cole *et al*., 2008; see Methods S1) were used to help initially identify and collect individuals of

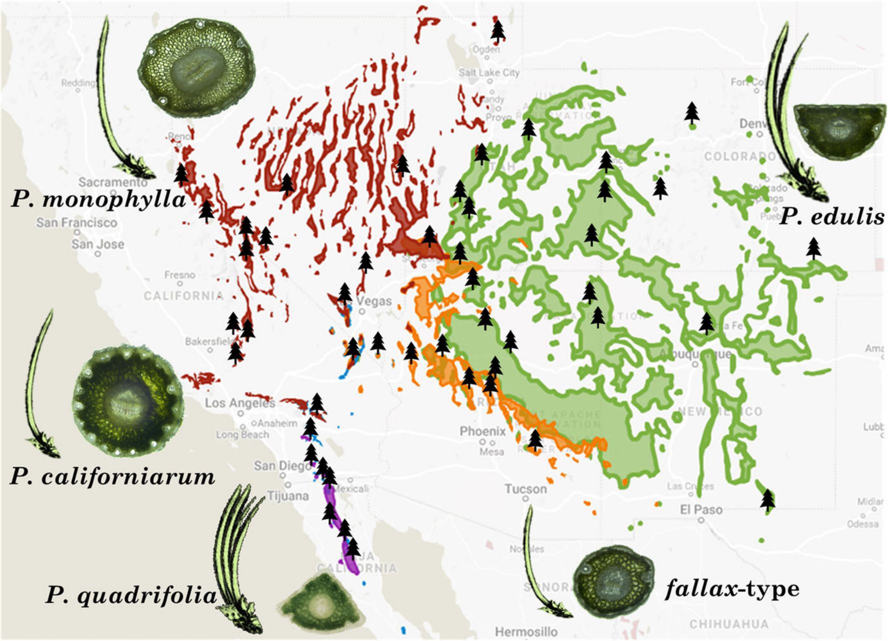

Fig. 1 Distribution map colored by morphotypes identified in Cole *et al*. (2008): *Pinus monophylla* (red), *Pinus edulis* (green), *Pinus quadrifolia* (purple), *Pinus californiarum* (blue), and *fallax*-type (orange; based on Little, 1999; Cole *et al*., 2008). Superimposed images display the number of needles per fascicle and leaf cross section, showing its typical shape and the number of resin canals (ducts in the periphery of the leaf). Sampling populations are represented as black tree symbols.

all taxa across their distribution, multidisciplinary approaches including genetic methods, niche modeling, morphology, and geography were later employed to reconcile species boundaries (Curtu *et al*., 2007; Leaché *et al*., 2009; Lissambou *et al*., 2019). Initial samples included 276 *Pinus edulis* Engelmann, 203 *P. monophylla* Torrey and Fremont, 200 *P. californiarum* Bailey, 130 *fallax*-type, 121 *P. quadrifolia* Parlatore ex Sudworth, and 33 hybrid morphotypes. All 174 samples used in the Buck *et al*. (2020) study were used here, adding two more taxa (*P. edulis* and *fallax*-type) and 35 populations, for a total of 789 more samples. Ten centimeters of branch tips were cut from each tree, representing 2–3 yr of growth, and averaging *c*. 50 fascicles per tree.

Nuclear DNA sequencing

Nuclear DNA was extracted using Doyle & Doyle's (1987) CTAB 2% protocol, quantified, and sent to Diversity Arrays Technology (DArT), who use genome reduction methods and next-generation sequencing platforms similar to ddRAD-seq but to target low-copy fragments (Jaccoud *et al*., 2001). This method produces a higher number of informative single nucleotide polymorphisms (SNPs) across the genome that are then compared with a reference genome (Buck *et al*., 2020). We selected *P. lambertiana* as the reference genome (GCA_001447015.2) because of its relatively close evolutionary relationship with the pinyon subsection *Cembroides* (Montes *et al*., 2019; Jin *et al*., 2021) compared to that of *P. taeda*. A low-density DArTseq assay of 353 individuals (Table S1) subset from across the distribution (Fig. 2b) resulted in 24 109 SNPs, coded as '0' (homozygous to reference allele), '1' (homozygous to alternative allele), '2' (heterozygous), and '-' (fragment missing). Appropriate file types needed for downstream analyses were reformatted from the processed marker data using the R program DARTR (Gruber *et al*., 2018).

Genomic clustering analyses

From the original raw SNP dataset, SNPs were filtered to remove all loci with > 10% missing data, all monomorphs, all loci with a reproducibility quality score lower than 1, all loci departing from Hardy–Weinberg equilibrium, and all but one locus where there was more than one locus per sequence tag, resulting in a final cleaned dataset of 1891 loci and 353 individuals. We analyzed patterns of population structure among individuals using a principal coordinate analysis (PCoA) with DARTR using the gl.pcoa.plot command and 95% confidence intervals (Gruber *et al*., 2019). Additionally, we determined population clusters in the Bayesian software FASTSTRUCTURE (Raj *et al*., 2014) using the logistic prior and 10 cross-validations. The chooseK command was used to select model complexity (K). The resulting Q mean bar plots were visualized using the online application POPHELPER (Francis, 2017). Population clustering was further assessed using Admixture (Alexander *et al*., 2009) and a discriminant analysis of principal components (DAPC; Jombart *et al*., 2010; Methods S2).

Genetic differentiation analyses

F_{st} As hybridization can bias fixation index results (Nei, 1986), we separated the genetically uniform individuals from the admixed individuals identified in our FASTSTRUCTURE analyses (Q score < 1) and used the stamppFst command in the R package *ADEGENET* (Jombart, 2008) to calculate the F statistic, applying 1000 bootstraps and a 95% confidence interval.

Heterozygosity In order to estimate the genetic diversity of each taxon, we calculated the heterozygosity of each taxon, using the same dataset as in F_{st}, and implemented the gl.report.heterozygosity command in the *ADEGENET* package in R.

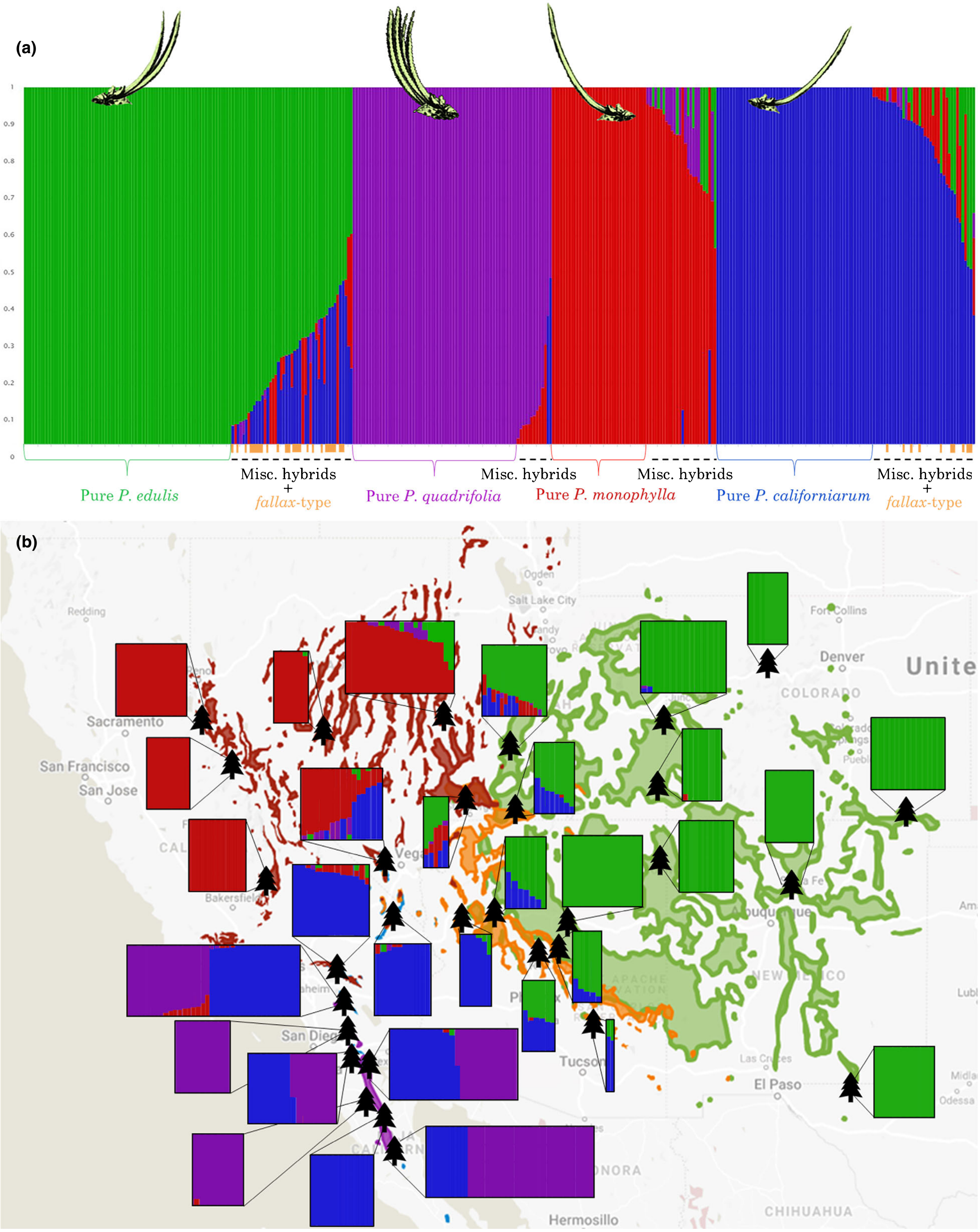

Fig. 2 FastStructure plot showing four genetic clusters with extensive admixture among all species. (a) Each vertical line represents an individual, with the color of the line illustrating the probability of that individual belonging to a certain genetic cluster. Lines with a single color represent 'genetically uniform' individuals belonging to only one genetic cluster, while lines with multiple colors represent admixed individuals belonging to multiple genetic clusters. *Fallax*-type individuals are generally composed of only green and blue admixed bars. Superimposed figures represent the typical needle number associated with each genetic cluster. (b) FastStructure plots on the distribution map by population showing admixture in areas of sympatry. Clusters are colored by genetic identity: green, *Pinus edulis*; purple, *Pinus quadrifolia*; red, *Pinus monophylla*; blue, *Pinus californiarum*. Individuals morphologically identified as *fallax*-type appear as admixed individuals of *P. edulis* and *P. californiarum*. Sampling populations are represented as black tree symbols.

Hybrid generation identification

NewHybrids We applied a Bayesian model-based clustering method to determine each individual's hybrid category in the software NewHybrids 1.0 (Anderson & Thompson, 2002), which uses a Markov chain Monte Carlo method to compute the posterior probability of an individual belonging to predefined ancestry categories (Table S2). The program only compares two parental genotypes at a time, so we created six datasets to represent each pairwise species cross by removing individuals with ancestry of the third and fourth species. We initiated runs at different random starting points with a burn-in of 10 000 and 100 000 sweeps, using Jeffrey's prior for both pi and theta (Couch *et al.*, 2016).

Demographic parameter estimation

Fastsimcoal We tested 11 demographic scenarios (Fig. S1; Methods S3) in Fastsimcoal2.6 (Excoffier & Foll, 2011; Excoffier *et al.*, 2013). To increase the SNP count required for the analysis, we created a modified SNP dataset (Methods S3) and used easySFS (https://github.com/isaacovercast/easySFS) to generate a folded multidimensional site frequency spectrum. Each demographic model was replicated 100 times using 40 expectation–conditional maximization (ECM) cycles, a log precision of 18, and 200 000 simulations to calculate the likelihood. A generation time range of 40–80 yr was used to convert output parameter estimates (e.g. 100 generations = 4000–8000 yr ago), as these trees begin producing female cones at *c.* 40-yr-old and become sexually mature at *c.* 80 (Ronco Jr, 1990; Cobb *et al.*, 2002) and a mutation rate of 8.04×10^{-8} per site per generation was implemented as an average of the reported nuclear mutation rate for *Pinus* (Willyard *et al.*, 2007; 1.005×10^{-9} per site $\mathrm{yr}^{-1} \times 80$ yr per generation). We used Akaike's information criterion (AIC) to find the most probable model given the observed data. Confidence intervals on parameter estimates for the best-supported model were generated via block bootstrapping by randomly sampling 100 blocks of the SNP dataset and creating 100 new SFS per model in easySFS. These 100 SFS were then run 50 times each in Fastsimcoal2 using the same parameters and conditions as the original runs for both of the best models. The estimates from the best runs (highest likelihood) out of the replicated 50 were then used as confidence intervals, removing the lowest and highest five estimates for each parameter. These analyses took 417 d of run-time using 356 processors on two computing clusters: UCR's High-Performing Computing Center (HPCC) and UNAM's Laboratorio Nacional de Visualización Científica Avanzada.

Chloroplast DNA analysis

Microsatellites and multiplex design A total of 18 chloroplast microsatellite markers developed for subsection *Cembroides* (Flores-Rentería & Whipple, 2011) were considered in this study due to the lack of variation among chloroplasts. Amplification was done from DNA of *P. edulis*, *P. monophylla*, *P. quadrifolia*, *P. californiarum*, and *fallax-type* (Table S1). Four of 14 markers were excluded due to homoplasy (compound microsatellite 102213), lack of variation (61350, 108909), or other causes (108297). Additional markers (26081, 72502, 15147, 48509, and 79293) were developed following Flores-Rentería & Whipple's (2011) protocol, which reduces stutter in mononucleotide repeats. Multiplex design, PCR conditions, and subsequent genotyping analyses were performed as described in Flores-Rentería *et al.* (2013).

Principal component analysis In order to visualize genetic variation among chlorotypes, we ran a principal component analysis (PCA) in R using the adegenet package. The resulting PCA was plotted using the ggplot2 package, coloring individuals based on their nuclear genetic clustering identified in FastStructure.

TCS In order to create a haplotype network, a distance matrix was input into the software TCS (Clement *et al.*, 2000) using an 11-step limit to connect all haplotypes. The network was then visualized using tcsBU (Múrias dos Santos *et al.*, 2016).

Niche divergence

MaxEnt To model the current habitat suitability of the taxa in this study, environmental niche models were generated using MaxEnt v.3.4.4 (Phillips *et al.*, 2006). The original 30 arc-second variables used to build the suitability models were 19 bioclimatic variables and an elevation raster from WorldClim2 (Fick & Hijmans, 2017), and 36 categorical soil classes, eight slope classes, and five slope aspect classes from the Harmonized World Soil Database (Fischer *et al.*, 2008) and cropped to encompass the study area containing the observation points (Methods S4), resulting in a final observation count of 1449 for *P. monophylla*, 1353 for *P. edulis*, 1343 for *P. californiarum*, 745 for *P. quadrifolia*, and 172 for *fallax*-type. Independent models were created for each species by removing correlated variables ($|r| > 0.70$; Merow *et al.*, 2013) and those with 0% contribution to the model, and using 10 bootstrapped replicates, jackknifing, a random test percentage of 30 (Hernández-Quiroz *et al.*, 2018), a maximum of 5000 iterations, a 1.5 regularization multiplier (Merow *et al.*, 2013), and a 10 percentile training presence.

ENMTools To determine whether niche models of taxa are divergent, niche models were compared in ENMTools 1.4 (Warren *et al.*, 2008, 2010) using the overlap test and either the identity test for species with overlapping distributions (*P. edulis* and *P. fallax*-type, *P. quadrifolia*, and *P. californiarum*) or the background test for allopatric species (all other species pairs; Warren *et al.*, 2008). In the overlap test, a *D* value for each pairwise comparison was produced using the average rasters created in the MaxEnt runs above. For the identity tests, pairwise comparisons using 100 replicates and all input occurrence points generated a distribution of *D* values that were then compared with the *D* value from the overlap test. For the background tests, pairwise comparisons using 100 replicates and a background raster with a 30 km-buffer around occurrence points generated a distribution of D values that were then compared with the D value from the overlap test.

Morphology

Due to the inherent biases that arise with morphological classification and the already existing taxonomic controversies in subsection *Cembroides* (Price *et al.*, 1998; Cole *et al.*, 2008; Buck *et al.*, 2020), we first assigned samples to genetic groups and then determined what, if any, morphological traits were consistent within each group, or whether intermediate morphology was associated with hybridization. We analyzed a range of leaf morphological traits typical to pine analyses (Little, 1968; Bailey, 1987; Lanner & Phillips, 1992; Malusa, 1992; Christensen *et al.*, 1995; Cole *et al.*, 2008; Flores-Rentería *et al.*, 2013) following the protocol used in Buck *et al.* (2020). We used an ultrahigh-resolution Nikon SMZ25 stereoscopic microscope zoom ×0.5–1.6 and NIS Elements software for all measurements. We also ran a PCA analysis to visualize morphological differences among the taxa and estimate the contribution of each variable to the separation, normalizing the data using the R package *BESTNORMALIZE*.

Results

Genetic structure

Our nuclear genetic clustering and admixture analyses on 353 individuals with 1891 SNPs show that *P. edulis* and *fallax*-type participate in the syngameon.

Our FASTSTRUCTURE analyses (Figs 2a, S2) visualized in POPHELPER (Francis, 2017) and superimposed onto the distribution map to help discern the structure of the syngameon show four distinct genetic clusters that correspond to previously hypothesized taxa: *P. edulis*, *P. quadrifolia*, *P. monophylla*, and *P. californiarum*, supported by both the DAPC (Fig. S3) and Admixture results (Fig. S4). Extensive admixture among all species is observed, with most admixed individuals appearing near areas of sympatry, while genetically-uniform individuals exist at the range cores (Fig. 2b). Notably, the individuals identified as *fallax*-type show up as admixed between *P. edulis* and *P. californiarum*.

Our PCoA (Fig. 3a) showed four groups of individuals composed of *P. monophylla, P. quadrifolia*, *P. edulis*, and *P. californiarum*. PCoA axis 1 accounted for 14.9% of the variation, while axis 2 accounted for 6.1% of the variation. When admixed individuals were included (Fig. 3b), *fallax*-type was found to overlap *P. edulis* and *P. californiarum*. PCoA axis 1 accounted for 13.2% of the variation and matches the west-to-east distribution of the taxa, while axis 2 accounted for 5.2% of the variation and matches the south-to-north distribution of the taxa.

Genetic differentiation

Our F_{st} results (Fig. 4b) indicated low differentiation between *fallax*-type and *P. californiarum* and *fallax*-type and *P. edulis*. Hybridization is expected to increase genetic diversity; therefore, we estimated heterozygosity as a measure of genetic diversity among taxa. The heterozygosity of each taxa was as follows: *P. quadrifolia* 0.0242, *P. edulis* 0.0269, *fallax*-type 0.0350, admixed individuals 0.0401, *P. monophylla* 0.0405, and *P. californiarum* 0.0479.

Chloroplast capture

As the chloroplast is inherited paternally in most pines (Neale & Sederoff, 1989; Kormutak *et al.*, 2018), if two species hybridize and subsequently backcross, one parental species can acquire the chloroplast of the other parental species, a process known as chloroplast capture. The comparison of nuclear and chloroplast data can aid in the understanding of parental contribution and the detection of chloroplast capture events and cryptic hybrids. In the chloroplast PCA analysis (Fig. 5), we colored individuals based on their main nuclear genetic clustering in FASTSTRUCTURE and found three groups. *Pinus monophylla* and *P. quadrifolia* had distinct chlorotypes, while *P. edulis*, *P. californiarum*, and *fallax*-type share a similar chlorotype. One admixed individual with the nuclear identity of *P. edulis* grouped with the *P. monophylla* chlorotype (Fig. 5a, asterisk). Similarly, several admixed individuals with the main nuclear identity of *P. monophylla* grouped with the *P. edulis-P. californiarum-fallax-type* chlorotype (Fig. 5a, arrows) and the *P. quadrifolia* chlorotype (Fig. 5a, X). All of these individuals originate from the contact zones where *P. edulis* and *P. monophylla* overlap in distribution, suggesting chloroplast capture events. It is important to note that because the chloroplast is inherited as a whole, it is considered a single gene and therefore many of the loci used could be highly correlated. However, despite this property, both the PCA and the haplotype network (Fig. S5) show similar patterns, with *P. edulis*, *P. californiarum*, and *fallax*-type individuals having indistinguishable haplotypes.

Demography

In order to understand the temporal structure of this syngameon and estimate the direction and magnitude of gene flow, we compared 11 demographic scenarios (Fig. S1). The two scenarios that best explain our data (Fig. 4a) revealed a recent hybrid speciation event, with either *P. quadrifolia* or *P. monophylla* hybridizing with *P. edulis* 72 000–150 000 yr ago to create *P. californiarum*; however, the AIC scores were too close to determine which is the best model (Table S3). The discrepancies in parameter estimates between the models are likely due to the limitations of site frequency spectra, where demographic histories with very different parameter estimates can give the same site frequency patterns (Meyers *et al.*, 2008). Extensive gene flow among all species was detected in the present time (Table S4), corroborating the syngameonic behavior found in the FASTSTRUCTURE results.

Hybrid generation

We assessed whether the detected hybrid populations including *fallax-type* are recent and recurring F_1s or are stable lineages

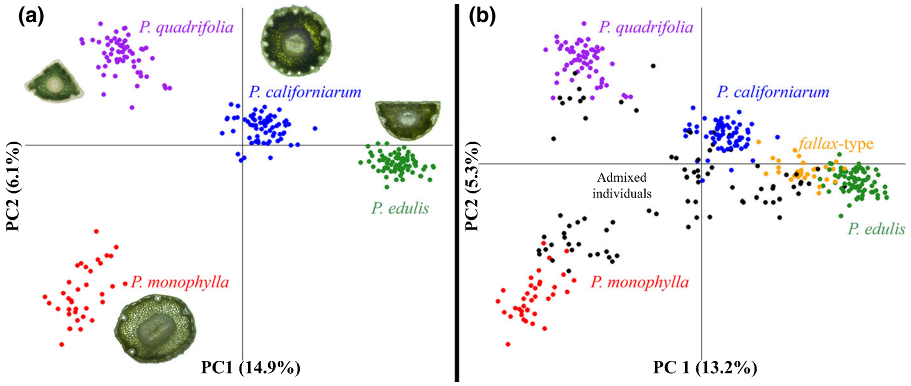

Fig. 3 Principal coordinate analysis using 1891 unlinked nuclear single nucleotide polymorphisms (SNPs) showing the variation in genetic distances among the four main groups with (a) all admixed individuals identified in FastStructure removed and (b) all individuals present. Black dots correspond to individuals with hybrid ancestry identified in FastStructure. Individuals forming *fallax*-type emerge between *Pinus edulis* and *Pinus californiarum*. Images represent the typical leaf morphology and relative size for each genetic group.

with advanced generation admixed individuals using the software NewHybrids (Anderson & Thompson, 2002) with nuclear data. The hybrid generations determined by NewHybrids (Table S5) showed most of the admixed individuals identified in FastStructure as advanced generation backcrosses, with 20 individuals identified as fourth generation or further. One hundred individuals had lower confidence in their generation assignment, potentially due to the pairwise limitations of NewHybrids, or that they are beyond the generation categories tested.

Niche divergence

To explore whether the hybrid origins of *P. californiarum* and *fallax*-type could have been driven by differing niches, habitat suitability models were generated with an AUC score > 0.9, suggesting they are useful models (Elith, 2000; Phillips & Dudík, 2008). The suitability models indicated the niche of *P. californiarum* was divergent from *P. edulis* and *P. quadrifolia*, with potential divergence from *fallax*-type and *P. monophylla*. *Pinus monophylla* had a divergent niche from *P. edulis*, with potential divergence from *P. quadrifolia*. *Fallax*-type showed a niche divergent from *P. edulis* and *P. monophylla*, with a potential divergence from *P. quadrifolia* (Fig. S6).

Morphology

In order to determine whether genetic clusters had identifiable morphological characteristics, we examined several needle traits. Our morphological analyses (Figs 5b, S7) revealed that species in nonadmixed populations can typically be identified by using the number of needles per fascicle, stomatal rows, and resin canals together. Individuals from genetically uniform *P. monophylla* descent tend to have one needle per fascicle with two to four resin canals and 20–31 stomatal rows, mainly distributed in Central and Northern California, Nevada, and parts of Western Utah. *Pinus monophylla* and *P. californiarum* overlap extensively when examining a multitude of traits (Fig. 3c); however, the two species can be distinguished using the number of resin canals and stomatal rows (Fig. S7a,c, in agreement with Cole *et al.*, 2008). Genetically uniform *P. californiarum* individuals generally have one needle per fascicle with eight to 14 resin canals and 15–23 stomatal rows, observed mostly in Southern California, Baja California Norte, and the southern tip of Nevada. Individuals from genetically uniform *P. quadrifolia* typically have four to five needles per fascicle (but individuals with three needles have been observed) with two resin canals and six to nine stomatal rows, mainly distributed in Southern California and Baja California Norte. Genetically uniform *P. edulis* individuals usually have two needles per fascicle with one to two resin canals and nine to 12 stomatal rows, observed mostly in Colorado, Utah, New Mexico, and Arizona. *Fallax*-type has a varying morphology falling between *Pinus californiarum* and *P. edulis* (Fig. S7a,c), with zero to eight resin canals and 12–17 stomatal rows, depending on the contribution of each parent. Individuals of *fallax*-type are mainly distributed in Western and Southern Arizona, along the Mogollon Rim. Admixed individuals tend to have the morphology of the parent that contributes more to the genome, especially ones identified as advanced generation backcrosses (Fig. S7b,d).

Discussion

Our results showed that hybridization is widespread and has played an important role in the pinyon pine complex. This is the first study documenting sequential hybridization events in natural systems, in which a novel independent lineage resulting from gene flow (*P. californiarum*) hybridized with one of its parents to create a second novel lineage (*fallax*-type; Fig. 4b).

Syngameonic hybridization restricted to range edges

In order to understand the dynamics and structures of syngameons, it is crucial to determine what species are participating in them and how those species are connected spatially and through gene flow. Our genetic and morphologic data suggest *P. edulis*, *P. monophylla*, *P. quadrifolia*, and *P. californiarum* are distinct species that all hybridize among each other. The inclusion of *P. edulis* in this study resulted in the discovery of its participation in this known syngameon, having extensive admixture with the three described species, especially with *P. monophylla* and *P. californiarum*. Admixed individuals in this syngameon are generally restricted to areas of sympatry at the edges of the participating species' ranges, suggesting that these patterns are not produced by incomplete lineage sorting, supporting the range-edge

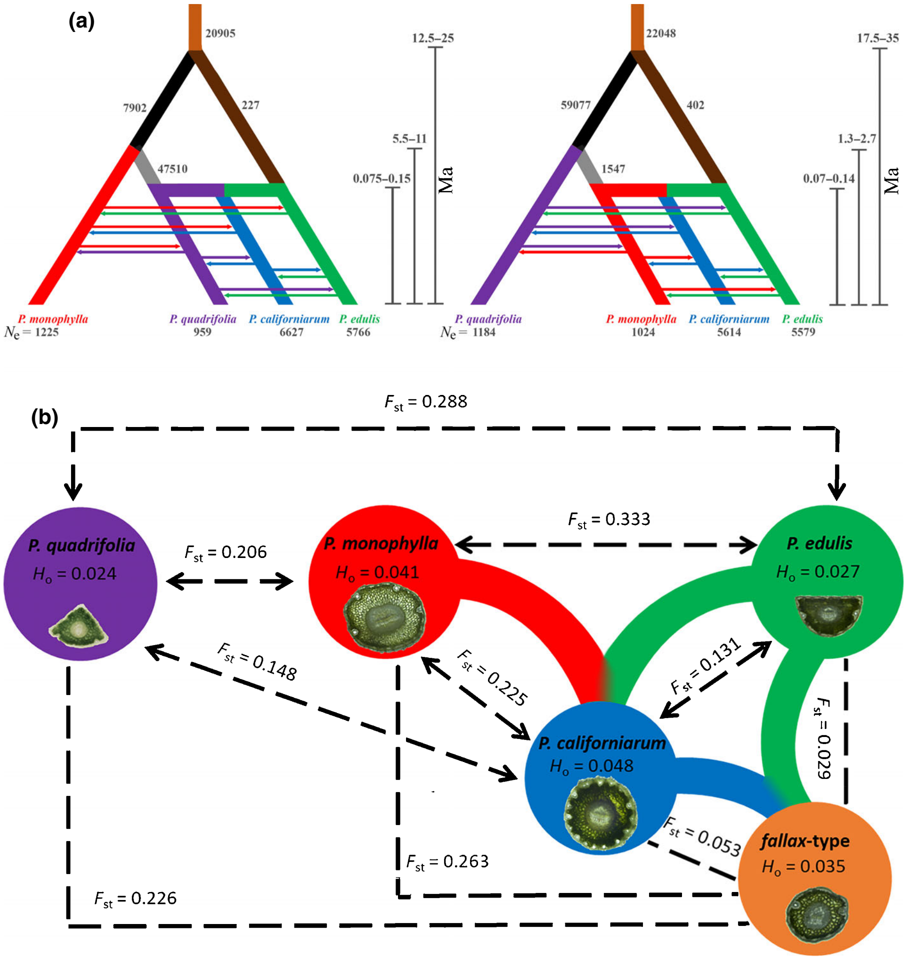

Fig. 4 Hybridization has played a role in the speciation process in pinyon pines. (a) The two most likely FASTSIMCOAL scenarios determined by Akaike's information criterion (AIC) calculation showing the hybrid origin of *Pinus californiarum* from *Pinus edulis* and either *Pinus monophylla* or *Pinus quadrifolia*. Estimated effective population sizes are represented by the numbers on the sides of the models, while estimated divergence times are reported in bars to the right of each model. (b) Sequential hybridization diagram showing the hybrid origins of *P. californiarum* from *P. monophylla* and *P. edulis* and of *fallax*-type from *P. californiarum* and *P. edulis*. Dashed lines with arrows represent gene flow connections, with population differentiation (F_{st}) estimates shown above all dashed lines. Heterozygosity estimates are shown within each taxon's circle, along with an image of a needle cross section typical for the taxon. Ma, million years ago.

formation hypothesis (Cronk & Suarez-Gonzalez, 2018), and giving insight into the structure of this syngameon. The lack of introgression further into the species' cores could explain how they can participate in the syngameon yet maintain their species' identities. While adaptive alleles formed in the hybrid zones may not be introgressed far into species' range cores, they are important drivers in range expansion, especially into new, more extreme environments on the edge of tolerances for core populations (Pfennig *et al.*, 2016). Usually, a narrow hybrid zone is a result of postzygotic isolating mechanisms (Barton & Hewitt, 1985); however, our NEWHYBRIDS analyses showed that many individuals appear to be a result of several generations of admixture, implying that F_1s and F_2s are able to form, are viable, and are fertile enough to mate with each other and backcross with the parental species. Further, these pines lack genetic isolating mechanisms (Critchfield, 1975), and have overlapping pollen dispersal times (Malusa, 1992; Farjon & Styles, 1997). This may mean that there are no discernable intrinsic reproductive isolating barriers present among these species of pinyon pines and that extrinsic factors such as climate are more important in isolating species (Cole *et al.*, 2008; Menon *et al.*, 2018).

These patterns of restricted hybridization could be a result of strong selective pressures against the parental species in the edges of their ranges where hybrids may have a selective advantage (Pfennig *et al.*, 2016), resulting in the replacement and local extinction of pure parental genotypes, that is, genetic swamping (Todesco *et al.*, 2016). This genetic swamping could explain the patterns seen in a few isolated desert populations at the furthest range edges of *P. monophylla*, *P. californiarum*, and *fallax*-type that consist of mainly admixed individuals and few, if any, parental individuals, and have significantly hotter and drier conditions. At the same time, there could be strong extrinsic factors selecting against unfit hybrids toward the core of the parental range, resulting in the local extinction of hybrids and the rarer parental lineage, that is, demographic swamping (Todesco *et al.*, 2016), preventing further introgression and interspecific migration into range cores.

Hybrid origins of *P. californiarum*

Pinus edulis and *P. californiarum* shared a similar chlorotype, hinting at a possible shared ancestry or chloroplast capture event. This led to our exploration of demographic scenarios in which *P. californiarum* was of hybrid origin as well. Our two most likely models supported the chloroplast data, showing that *P. californiarum* was indeed a hybrid species from *P. edulis* and either *P. monophylla* or *P. quadrifolia*. Although not all members of the pinyon subsection *Cembroides* were included in this study, the

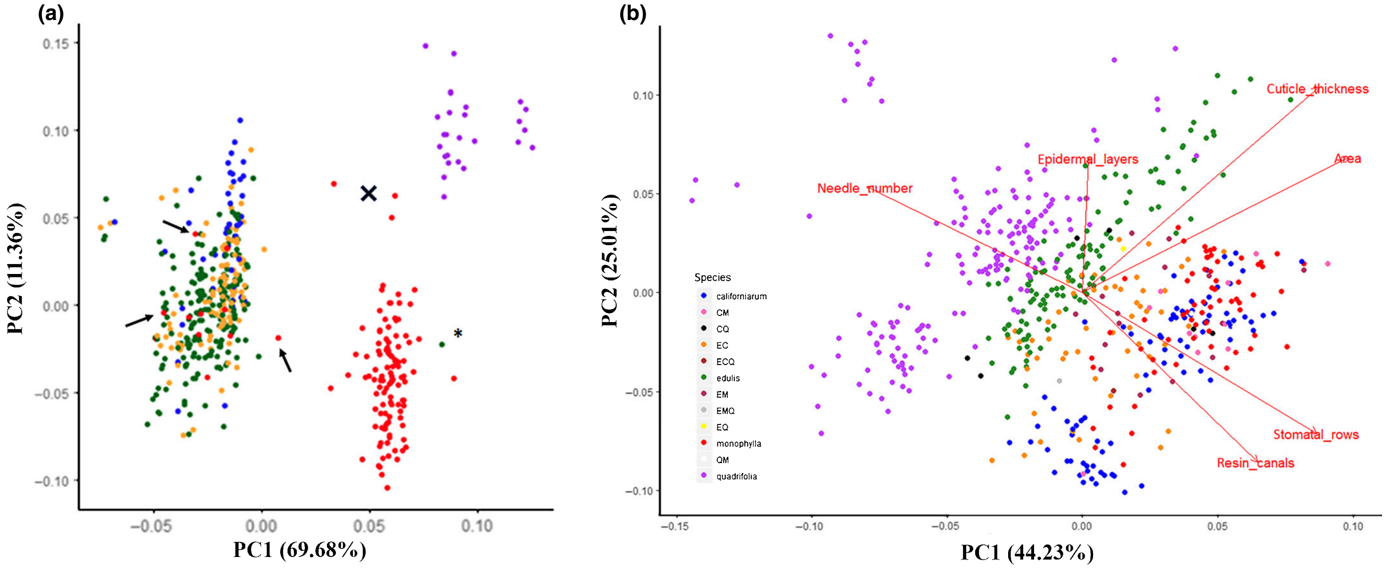

Fig. 5 Chloroplast and morphology data show patterns of hybridization and chloroplast capture on *Pinus edulis* (green), *Pinus californiarum* (blue), *fallax*-type (orange), *Pinus monophylla* (red), and *Pinus quadrifolia* (purple), with individuals represented as dots. (a) Principal component (PC) analysis using 18 chloroplast markers, suggesting *P. edulis*, *P. californiarum*, and *fallax*-type share a similar chloroplast, while *P. monophylla* and *P. quadrifolia* are distinct. Dots are colored by their main nuclear identity. One individual that had the nuclear identity of *P. edulis* had the chloroplast of *P. monophylla* (asterisk). By contrast, some trees that had the nuclear identity of *P. monophylla* had the chloroplast of *P. edulis*/*fallax*-type/*P. californiarum* (some examples represented by arrows) and *P. quadrifolia* (X mark), suggesting chloroplast capture events. (b) Principal component analysis of the leaf morphology among species colored by nuclear genetic identity. The red lines indicate component loadings, which are correlations between the original variables and the principal components that can help determine which variables are responsible for data variation. Hybrid individuals are represented as *P. californiarum* × *P. monophylla* (CM), *P. californiarum* × *P. quadrifolia* (CQ), *P. edulis* × *P. californiarum* (EC), *P. edulis* × *P. californiarum* × *P. quadrifolia* (ECQ), *P. edulis* × *P. monophylla* (EM), *P. edulis* × *P. monophylla* × *P. quadrifolia* (EMQ), *P. edulis* × *P. quadrifolia* (EQ), and *P. quadrifolia* × *P. monophylla* (QM).

inclusion of an additional species is not expected to change the demographic results, as an unsampled 'ghost' lineage was accounted for in the tested models (Fig. S1, models nos. 7–10). While extensive genomic studies that incorporate wide, population-level sampling can be costly, not including hybridizing members of a syngameon can limit the understanding of the evolutionary history and structure of not only the syngameon, but also each participating species. The suitability models indicated the niche of *P. californiarum* was divergent from *P. edulis* and *P. quadrifolia*, with potential divergence from *fallax*-type and *P. monophylla*. If *P. californiarum* and *fallax*-type do have niche divergence, then ecological differentiation could be driving the incipient speciation processes in *fallax*-type as a result of competition avoidance, hybrid superiority, or the colonization of a new niche. If they do not have niche divergence, the similarities could be a result of *P. californiarum*'s genetic influence on *fallax*-type or the partial, but not complete, ecological divergence of the two taxa. Alternatively, the inconclusive divergence results could signify the occupation of an intermediate niche; however, due to potential limitations in detecting niche intermediacy when using pairwise comparisons between hybrids and their progenitors, further comparisons looking at niche additivity must be done to support intermediacy (Marchant *et al.*, 2016; Parisod & Broennimann, 2016). *Pinus monophylla* had a divergent niche from *P. edulis*, with potential divergence from *P. quadrifolia*. *Fallax*-type showed a niche divergent from *P. edulis* and *P. monophylla*, with a potential divergence from *P. quadrifolia* (Fig. S6). While *P. edulis* and *fallax*-type partially overlap in distribution, they are separated by important ecological factors that may be driving *fallax*-type incipient speciation processes. The alternative model testing the origin of *P. californiarum* from a *P. monophylla* × *P. quadrifolia* hybridization event was not well supported (Fig. S1, model no. 6). The high rate of gene flow from *P. quadrifolia*/*P. monophylla* into *P. edulis* detected by the nuclear data coupled with the shared chlorotype data suggests that *P. edulis* was mainly a pollen donor while the hybrid descendants may have backcrossed multiple times with *P. quadrifolia*/*P. monophylla* to produce *P. californiarum*. The timing of this hybrid speciation event (72 000–150 000 yr ago) is also intriguing because that is a relatively short time (*c.* 1800 generations using a generation time of 80 yr) for pines to speciate (Menon *et al.*, 2018; Jin *et al.*, 2021), suggesting that hybridization may have accelerated the speciation process (Mallet, 2007; Abbott *et al.*, 2013). The estimated ancient distribution of these pines is restricted to 50 000 yr ago by the radiocarbon dating limit and the presence of packrat middens (Cole *et al.*, 2013), so we cannot be sure which species shared distributions during the creation of *P. californiarum*. While *P. monophylla* and *P. edulis* are thought to have begun overlapping in distribution at some time during the last glacial maximum 14 700–23 400 yr ago (Cole *et al.*, 2013), it is possible they were in contact at an earlier stage before migrating north. Unfortunately, not much is known about the ancient range of the more Mexico-distributed *P. quadrifolia* beyond 18 000 yr ago (Wells, 2000); however, *P. edulis*' distribution was considerably farther south than it currently is (Cole *et al.*, 2008, 2013; Duran *et al.*, 2012); therefore, it is possible that their ranges once overlapped or were at least close enough for pollen and seed dispersal (Wells, 1983; Williams, 2010). Future studies modeling climate beyond the reach of packrat midden data are needed to approximate ancient distributions and potential instances of sympatry. Hybridization with *P. monophylla* could explain the origin of its rare single-needle morphology or increased number of resin canals as a transgressive trait common in hybrid systems (Rieseberg *et al.*, 1999; Dittrich-Reed & Fitzpatrick, 2013). Further, a hybrid origin of *P. californiarum* could explain why it has the highest heterozygosity of any taxa, yet has the most restricted range. This inflated heterozygosity in turn could explain the large population sizes estimated for *P. californiarum*. Lastly, *P. californiarum* and *P. quadrifolia* often grow on the same mountain ranges often only miles apart in Southern California and Baja California, co-occurring in at least two locations (La Rumorosa and San Pedro Martir; Lanner, 1974b). This neighboring distribution could be a remnant of shared ancestry or a sign of homoploid speciation without allopatry, as demonstrated in other pines (Menon *et al.*, 2018).

Hybrid origins of *fallax*-type

Individuals of *fallax*-type, originally identified as having one needle per fascicle and two resin canals (Little, 1968), appear to be of hybrid descent, composed of both *P. edulis* and *P. californiarum*; therefore, we will hereby refer to it as *P.* ×*fallax*. This novel hybridization event was not detected in other genetic studies (i.e. Montes *et al.*, 2019, 2022) likely due to their lower sample sizes and taxonomically focused sampling design targeting 'type' individuals. However, the hybrid origin of *P.* ×*fallax* may explain their study's nuclear phylogenetic findings wherein *P.* ×*fallax* samples never formed a monophyletic group, but instead grouped with either *P. edulis* or *P. californiarum* (Montes *et al.*, 2022). As *P.* ×*fallax* is of hybrid origin, we expect to see its chlorotype grouping with either *P. edulis* or *P. californiarum* depending on the pollen donor; however, as *P. californiarum* likely inherited its chloroplast from *P. edulis*, the three taxa shared similar chlorotypes, while *P. quadrifolia* and *P. monophylla* remained distinct. A similar pattern using plastid data was found by Montes *et al.* (2022) in which *P. edulis*, *P.* ×*fallax*, and *P. californiarum* formed a monophyletic group together but individually had paraphyly, while *P. quadrifolia* and *P. monophylla* formed their own monophyletic groups.

Oddly, *P.* ×*fallax* was observed to have an intermediate level of heterozygosity between its two parental species, an unexpected observation of hybrid taxa. Typically, hybrids have higher levels of heterozygosity than their progenitors due to interspecific recombination (Fitzpatrick, 2012); however, selection, backcrossing, or a combination of the two could be responsible for *P.* ×*fallax*'s lower-than-expected levels of observed heterozygosity. If there are high selective pressures on *P.* ×*fallax*, alleles under selection and those neighboring them would be driven to fixation, reducing heterozygosity. Further tests measuring selective pressures and their effects on the pinyon genome are needed to support this hypothesis. Most *P.* ×*fallax* individuals were advanced generation backcrosses. High heterozygosity from hybridization would be expected in the first-generation hybrids (F_1) and even in the F_2s due to interspecific recombination; however, when backcrossing with parental species occurs, parental alleles are again recombined with the hybrid genomes, reducing heterozygosity with each backcross mating (vonHoldt *et al.*, 2013). Additionally, heterospecific parental alleles could be introgressed into each species through the hybrids, increasing the heterozygosity of the parental species, especially in sympatric populations at range edges.

Pinus edulis and *P. californiarum* have never been hypothesized to hybridize, probably due to the lack of both current overlapping distribution and samples with intermediate morphology, characteristics most authors have used to identify hybrids in this complex (Cole, 1956; Little, 1968; Lanner, 1974a,b; Lanner & Phillips, 1992). As the nonintermediate *P.* ×*fallax* morphotype has been found in middens from over 48 000 yr ago (Cole *et al.*, 2013), its stability likely also contributed to the lack of hybrid origin hypotheses. While F_1 hybrids of *P. monophylla* × *P. edulis* and *P. monophylla* × *P. quadrifolia* have consistent morphotypes (Critchfield in Lanner, 1974b; Buck *et al.*, 2020), further backcrossing reverts these later generation crosses to a parental phenotype (Buck *et al.*, 2020). This pattern of cryptic hybridization is also exemplified here (Notes S2), in which admixed individuals of later generations appear to have indistinguishable/similar morphology from their parents (Fig. 5b). However, *P.* ×*fallax* populations are mostly composed of advanced generation admixed individuals, yet they are still a constant and distinguishable lineage even though the parental species, *P. edulis* and *P. californiarum*, have no currently–overlapping distributions. The consistently forming morphology coupled with the uniquely hotter and drier niche that *P.* ×*fallax* occupies (Bailey, 1987; Cole *et al.*, 2008; Fig. S6) points to an incipient species with a possible selective advantage allowing individuals of hybrid ancestry from *P. edulis* and *P. californiarum* to inhabit a new arid, lower elevation environment. This process is similar to that observed in other hybridizing pine species in which the hybrid zones occur in a unique niche space that is hotter and drier than either parental species' habitats (Menon *et al.*, 2018). *Pinus* ×*fallax*'s ability to occupy this niche may be a result of the introgression of drought-adapted traits from *P. californiarum*. If so, these traits may also be introduced into *P. edulis* or the other species *P. californiarum* is hybridizing with and could potentially help them survive under future climate scenarios. This is especially important as *P. edulis* is a particularly sensitive indicator of climate change (Gitlin *et al.*, 2006) whose distribution has been susceptible to changes in climate for the last 40 000 yr (Betancourt, 1987, 1990). Future studies should focus on the fitness of *P.* ×*fallax* and examine adaptive introgression in the pinyon genomes.

Sequential hybridization

The implications of a hybrid speciation event in a syngameon give insight into the inner workings of a network undergoing frequent gene flow and emphasize the potential creative force of hybridization. *Pinus californiarum* inhabits a unique niche that is both drier and hotter than that of *P. monophylla* and *P. edulis* (Cole *et al.*, 2008; Ortiz-Medrano *et al.*, 2016). This suggests that the colonization of a new niche, specifically driven by drought stress tolerance, could have promoted the hybrid speciation event, similar to the process demonstrated in *Helianthus* (Rieseberg *et al.*, 2007) and other pines (Menon *et al.*, 2021). This type of hybrid speciation may be happening again with *P. californiarum* and *P. edulis* creating the morphologically stable *P.* ×*fallax*; however, the process has not yet been completed, as *P.* ×*fallax* does not have an independent genetic cluster and lower F_{st}. Further, pines have been described as diploid (Ledig, 1998; Grotkopp *et al.*, 2004; Ohri, 2021), meaning that *P. californiarum* could be an example of rare homoploid hybrid speciation (Goulet *et al.*, 2017). For some authors, hybrid speciation is hard to prove, especially in systems with ongoing gene flow, because complete reproductive isolation is not guaranteed. However, many agree (Mallet, 2007; Mavárez & Linares, 2008; Cannon, 2021) that complete reproductive isolation is not necessarily required for hybrid speciation as long as there are several lines of evidence, including genetic, morphological, geographic, and ecological separation, all of which are presented here or have been extensively documented for this group (Cole *et al.*, 2008, 2013; Ortiz-Medrano *et al.*, 2016). This is the first study that documents sequential hybridization events resulting in the formation of novel independent lineages that occupy a different ecological niche.

In the pinyon syngameon, species remain morphologically and genetically distinct at range cores and are able to maintain species boundaries while undergoing extensive gene flow in areas of sympatry at range peripheries. Additionally, the syngameon seems to be structured with several 'hub species' that contribute more genetic information than they receive and are connected to every other species through gene flow. Our study shows that hybridization has resulted in the formation of two new lineages in a relatively short time; thus, in this system, sequential hybridization may have accelerated the speciation process by providing a new combination of genes that allowed the colonization of different niches (Fig. S6). For example, *P. californiarum* which likely resulted from hybridization between *P. monophylla* and *P. edulis* retained one needle per fascicle but with fewer stomata rows. Both traits may be useful in colonizing drier and hotter environments, as drier environments co-occur with a decrease in needle number (Ortiz-Medrano *et al.*, 2016), and fewer stomata have been associated with drought tolerance (Knauf & Bilan, 1974; Guérin *et al.*, 2018). *Pinus californiarum* and *P. edulis* hybridize to create *P.* ×*fallax* individuals, and adaptive introgression from *P. californiarum* may allow *P.* ×*fallax* to colonize hotter and drier habitats. The shared chlorotypes of *P. edulis*, *P. californiarum*, and *P.* ×*fallax* led us to explore the hybrid origin of *P. californiarum*. The most likely models showed *P. californiarum* resulting from a hybrid speciation event between *P. edulis* and either *P. monophylla* or *P. quadrifolia*. Hybrid speciation could explain *P. californiarum*'s ability to occupy a unique niche, its transgressive resin canal number, its shared chlorotype with *P. edulis*, and its inflated heterozygosity despite having the most restricted range. The participation of *P. edulis* in this syngameon and the formation of two drought-adapted lineages begs larger questions about the stability and future of the pinyon syngameon. If one participating member goes extinct or develops reproductive barriers, will the structure remain intact or will the whole network collapse? If the network collapses, will the newly isolated species survive on their own, or was their dependence on the syngameon critical to their existence? Participation in the syngameon may allow adaptive traits to be introgressed across species barriers and provide the changes needed to survive under future climate scenarios.

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Author contributions

LF-R, RB and AVW designed the study. RB, LF-R, RM and BK collected plant material. LF-R, RB, RM and DF-R conducted lab work. LF-R, DO-DV and RB analyzed and interpreted data. RB and LF-R wrote the paper and made figures. LF-R, CG and AVW secured funding. LF-R, CG and AVW provided access to facilities. All authors provided feedback on the manuscript.

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Data availability

The data that support the findings of this study are openly available in Cyverse at https://data.cyverse.org/dav-anon/iplant/home/rcbuck/Fallax_paper_dataset.csv.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Topology of the 11 FASTSIMCOAL scenarios tested.

Fig. S2 FASTSTRUCTURE results showing *K* = 2–6.

Fig. S3 Discriminant analysis of principal component results showing *K* = 4.

Fig. S4 Admixture results showing *K* = 4.

Fig. S5 Chloroplast haplotype network.

Fig. S6 Niche divergence tests.

Fig. S7 Scatter plots of morphology with and without admixed individuals.

Methods S1 Sampling design.

Methods S2 Implementation of DAPC.

Methods S3 Design of FASTSIMCOAL scenarios and input data.

Methods S4 Design of niche model rasters and occurrence points.

Notes S1 Taxonomic information on subsection *Cembroides*.

Notes S2 Cryptic hybridization and syngameon detection.

Table S1 Sample locations ordered by longitude (west to east) with population coordinates and number of trees sampled per population and per analysis type.

Table S2 Twelve genotype frequency categories input into NEWHYBRIDS analyses.

Table S3 AIC scores of the 11 models.

Table S4 FASTSIMCOAL2 results.

Table S5 Levels of ancestry determined by NEWHYBRIDS for the hybrid derivatives.

See also the Commentary on this article by Whittemore & Miller, **237**: 1943–1945.