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Materials and Methods
Fig. S1
Tables S1 and S2
References

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Asynchronous Diversification in a Specialized Plant-Pollinator Mutualism

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Most flowering plants establish mutualistic associations with insect pollinators to facilitate sexual reproduction. However, the evolutionary processes that gave rise to these associations remain poorly understood. We reconstructed the times of divergence, diversification patterns, and interaction networks of a diverse group of specialized orchids and their bee pollinators. In contrast to a scenario of coevolution by race formation, we show that fragrance-producing orchids originated at least three times independently after their fragrance-collecting bee mutualists. Whereas orchid diversification has apparently tracked the diversification of orchids' bee pollinators, bees appear to have depended on the diverse chemical environment of neotropical forests. We corroborated this apparent asymmetrical dependency by simulating co-extinction cascades in real interaction networks that lacked reciprocal specialization. These results suggest that the diversification of insect-pollinated angiosperms may have been facilitated by the exploitation of preexisting sensory biases of insect pollinators.

The rapid diversification of angiosperms during the mid-Cretaceous, 90 to 125 million years ago (Ma), is often linked to mutualistic interactions with insect pollinators (*1*). Although fossil evidence suggests that generalist insect pollinators were present before the radiation of angiosperms (*1*, *2*), the more specialized flower-visiting (anthophilous) insects appear to have diversified synchronously with flowering plants (*1*–*3*). Today, more than 80% of angiosperms exhibit adaptations for insect pollination (*4*), and thus most terrestrial ecosystems depend on insect pollination services. However, our understanding of how plant-pollinator mutualisms evolved remains limited.

Because free-living mutualisms are embedded in complex webs of species interactions, understanding how adaptations emerge from—and contribute to—mutualistic associations remains a challenge in evolutionary biology (*5*). Darwin used coevolution (the idea that adaptive traits evolve by reciprocal selection) to explain and predict the existence of specialized traits exhibited by moth-orchid mutualisms in Madagascar (*6*). However, whereas reciprocal selection is now widely accepted as a driver of co-adaptation in antagonistic associations (e.g., between hosts and parasites), its role in free-living mutualisms such as plant-pollinator associations remains unclear (*7*, *8*). Because coevolution requires adaptive traits to evolve nearly simultaneously in both interacting lineages, we applied molecular phylogenetic techniques, molecular clock methods, chemical analyses, and network theory to infer whether reciprocal selection or one-sided selection shaped the evolution of a specialized plant-pollinator mutualism.

More than 200 species of euglossine bees in the neotropical region pollinate thousands of plant species while foraging for nectar, pollen, and resins (*9*, *10*). Additionally, male euglossine bees exhibit unique adaptations, such as specialized hind-leg pockets, for the acquisition and accumulation of fragrance compounds from flowers and other sources (*11*). During courtship display, male euglossine bees expose these fragrance mixtures. Although the full function of these fragrances has not been demonstrated, they are clearly involved in sexual selection, presumably by enabling species-specific recognition and/or acting as a signal of male fitness (*11*). The chemical composition of these fragrances evolves rapidly during lineage diversification, and one result may be the maintenance of reproductive isolation among closely related bee lineages (*12*). All species of euglossine bees exhibit these traits (*10*).

More than 600 species of orchids—equivalent to 10% of the neotropical Orchidaceae—have evolved adaptations for male euglossine pollination. Euglossine-pollinated orchids produce attractive volatile compounds (terpenes and aromatics), exhibit intricate mechanisms for the attachment of pollinaria (pollen masses) on male bees (Fig. 1A), and lack additional floral rewards such as nectar, pollen, or pseudopollen (*13*, *14*). It was previously thought that orchids depended exclusively on male bees for cross-fertilization and that male bees depended exclusively on orchid hosts for fragrance acquisition (and thus for access to mates) (*15*). However, recent observations suggest that although orchids do typically depend on euglossine bees for reproduction, male bees may obtain fragrance compounds similar to those produced by orchids from other hosts, including fungi, leaves, and rotting vegetation (*14*, *16*). Hence, it remains unclear whether coevolution shaped the mutualism between orchids and bees (*14*, *17*).

We reconstructed the phylogenetic relationships of Euglossini by sequencing ~4.0 kb of nuclear (*EF1-α*, *ArgK*, and *Pol-II*) and mitochondrial (*CO1*) DNA from 138 taxa sampled across the neotropical region (*18*, *19*). To estimate absolute divergence times among euglossine bee lineages, we used Bayesian relaxed- and strict-clock methods (*20*) and four different fossil calibrations (*19*). Our phylogenetic and dating analyses resolved most of the relationships within and between orchid bee lineages, recovered all genera as monophyletic, and indicated that euglossine bees shared a most recent common ancestor that lived during the Eocene-Oligocene, 34 to 38 (±4) Ma (Fig. 1 and Fig. 2). This age estimate concords with those obtained by previous studies (*3*, *18*).

To infer the phylogenetic relationships of euglossine-pollinated orchids and thereby establish bee-orchid associations, we collected orchid pollinaria directly from male bees captured in the field. We used synthetic fragrance baits to attract and screen >7000 individual bees (from >130 species; fig. S1). From these, we recovered a total of 193 pollinaria attached to ~40 bee species (figs. S2 to S6) and successfully sequenced ~3.0 kb of nuclear ribosomal (*ITS*) and chloroplast (*YCF1*) DNA from 148 samples.

Combined morphological and molecular data revealed that these samples comprised ~80 orchid species (13%) and 18 genera (51%), three of which had not been previously reported to exhibit male-euglossine pollination (table S2). Our phylogenetic analyses recovered three main clades of euglossine-pollinated orchids (Catasetinae, Stanhopeinae, and Zygopetalinae) and resolved most of the relationships within and between genera (Fig. 1B and figs. S7 and S8). We incorporated additional sequence data (*19*) of closely related orchids exhibiting food-deceptive pollination modes and confirmed that the three lineages of euglossine-pollinated orchids do not form a monophyletic clade (Fig. 1C) (*21*, *22*). Thus, a likelihood ancestral state reconstruction analysis suggested that switches from food-deceptive ancestors to male-euglossine pollination resulted in at least three independent radiations (Fig. 1C).

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Euglossine-pollinated orchids achieve reproductive isolation and avoid hybridization either by switching pollinators or by attaching pollinaria to different body parts of the same species (*14*). We investigated which mechanism appeared to contribute more to the diversification of orchid lineages by mapping these traits to the orchid phylogeny (Fig. 1A). Mapping pollinaria attachment sites on the orchid phylogeny (*19*) revealed a strong phylogenetic signal (Pagel's $\lambda = 0.93$, $P < 0.01$) and showed that attachment site shifts are more frequent between genera than within genera (Fig. 1A). An exception to this pattern is the recently diverged genus *Catasetum* (~4 to 11 Ma), which exhibited three alternative attachment sites on bees (Fig. 1A). In contrast, we observed no phylogenetic signal in the distribution of pollinator species used by orchid lineages (one-tailed Mantel test, $P = 0.76$). Because bee pollinators are known to finely partition chemical niche space (*12*), small changes in the chemical profile of floral scent can lead to changes of pollinator species, which can result in immediate reproductive isolation among orchid lineages. Our data suggest that chemically mediated shifts in pollinator species (as revealed by network connections) are more prevalent than the morphological changes required for shifting pollinaria placement (Fig. 1A).

We next estimated the absolute divergence times of euglossine-pollinated orchids using relaxed- and strict-clock Bayesian methods (*20*) and three fossil calibrations (*19*, *23*, *24*). Our estimates indicate that Catasetinae [18 to 27 (±4) Ma], Zygopetalinae [20 to 25 (±3) Ma], and Stanhopeinae [21 to 26 (±3) Ma] each shared most recent common ancestors that lived during the Oligocene-Miocene period (Fig. 2A). This result yielded substitution rates that approximately match those of other herbaceous plants (fig. S9).

Together with the bee divergence times, these age estimates revealed that fragrance collection in euglossine bees evolved at least ~12 million years earlier than fragrance production in euglossine-pollinated Orchidaceae (Fig. 2A). This result, along with the observation that male bees acquire fragrance compounds from non-orchid sources (*25*), suggests that the mutualism between euglossine bees and orchids originated as an asymmetrically dependent association in which euglossine-pollinated orchids independently adapted to, and exploited, a preexisting behavioral preference in bees. Although molecular clock estimates may suffer from biases due to incomplete lineage sampling, substitution rate heterogeneity, or sparse fossil data, our age estimates concord with those of previous studies (*3*, *18*, *23*, *26*). Thus, the asynchronous diversification of lineages in the free-living euglossine-orchid mutualism contrasts considerably with the simultaneous origin of lineages in obligate dependent mutualisms, including figs and fig wasps (*27*).

To test whether orchid lineages converged on preexisting chemosensory preferences of male bees, we determined the proportion of chemical compounds that the bees obtain from euglossine-pollinated orchids relative to non-orchid sources. We compared the chemical composition of (i) floral scents from 64 species of euglossine-pollinated

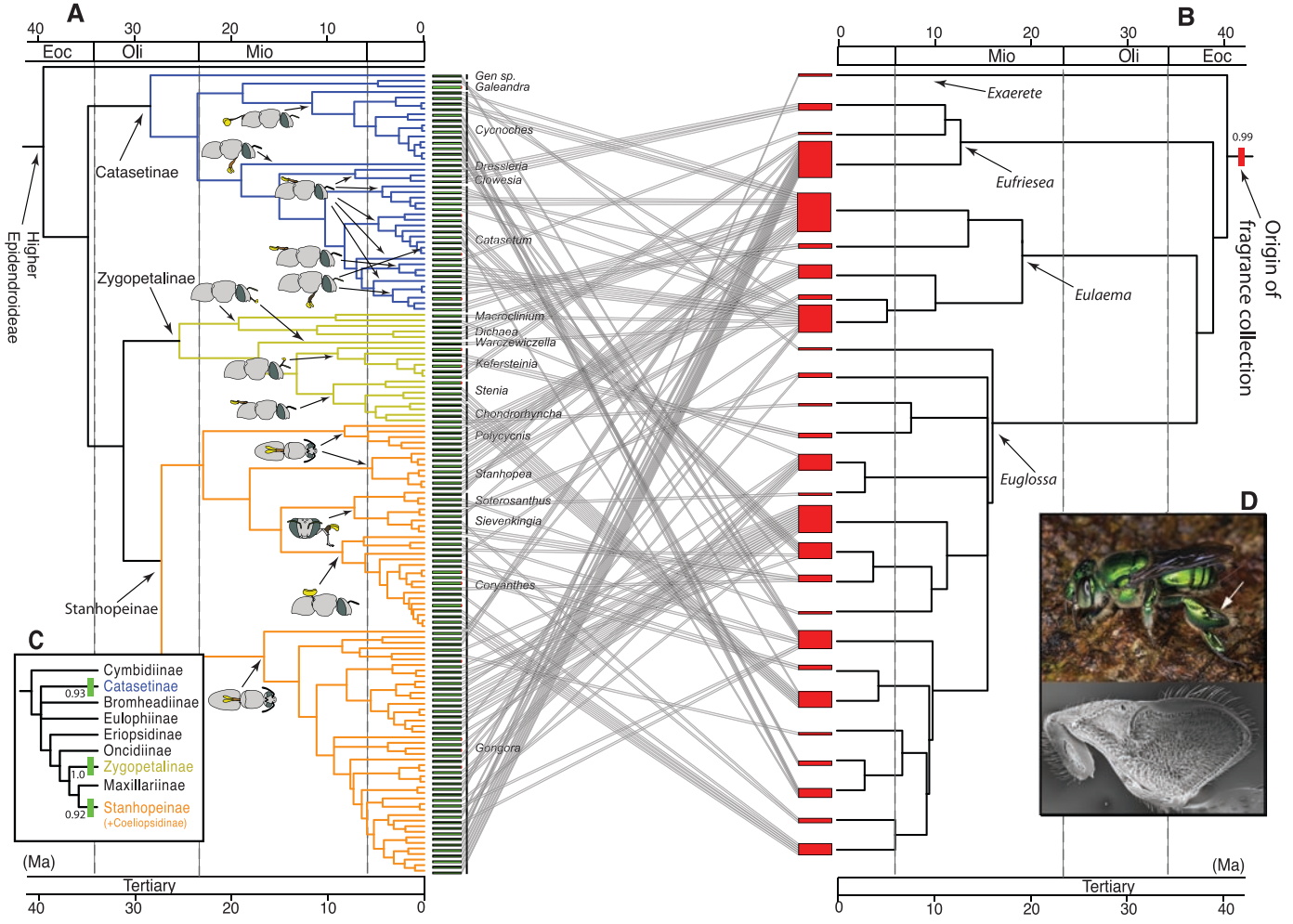

Fig. 1. (**A** and **B**) Fossil-calibrated chronograms of euglossine-pollinated orchids (A) and euglossine bees (B). Bipartite network and pollinaria attachment sites shown in (A) were inferred from pollinaria found attached to male bees. (**C**) The phylogenetic placement of orchid and bee lineages supports at least three independent origins of euglossine pollination (green bars) and a single origin of fragrance collection [red bar in (B)]; proportional likelihood values for reconstructed characters are shown. (**D**) Male *Euglossa* gathering fragrances (magnification 1.2×). Volatiles are stored in the tibial organ (arrow); scanning electron micrograph (magnification 8×) shows section of "pocket" on the bee leg where compounds are stored for subsequent use during courtship. [Upper photo: B. Jacobi]

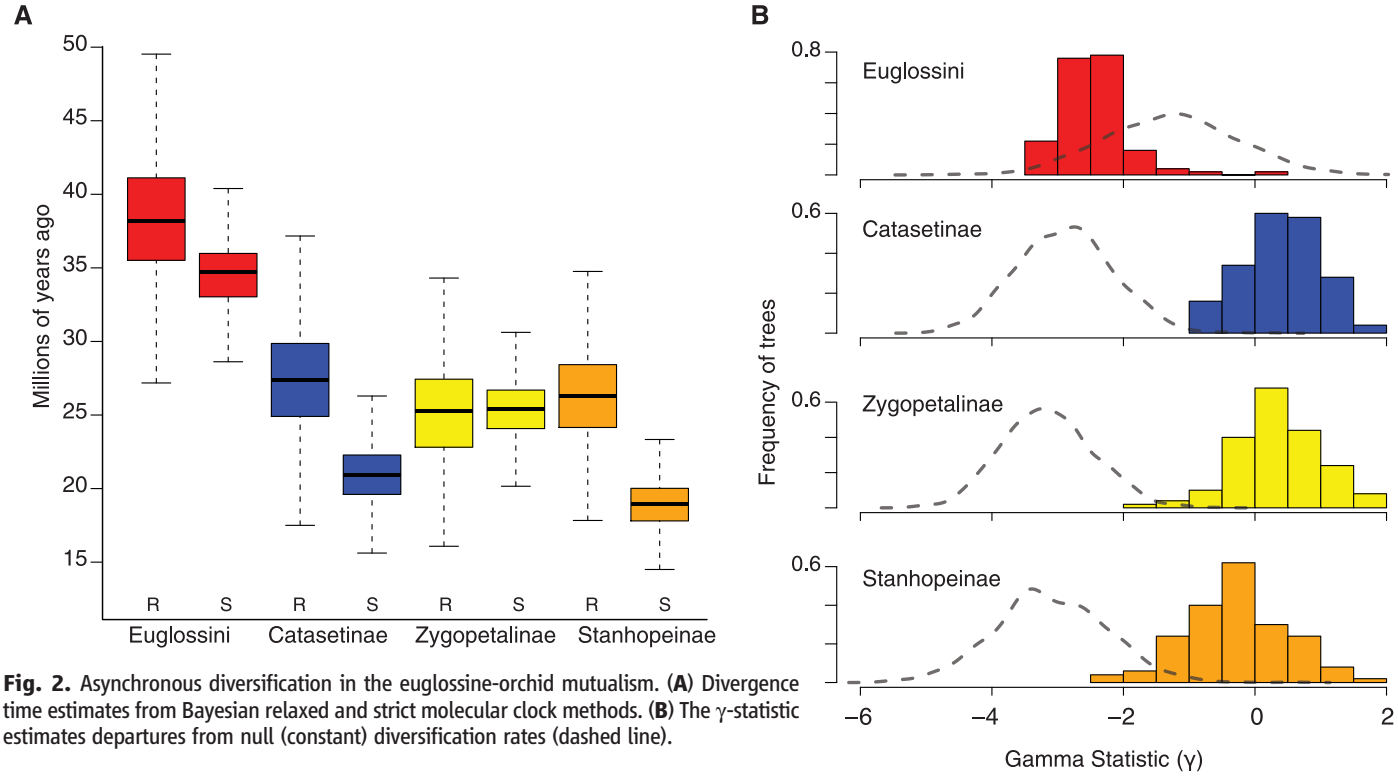

Fig. 2. Asynchronous diversification in the euglossine-orchid mutualism. (**A**) Divergence time estimates from Bayesian relaxed and strict molecular clock methods. (**B**) The γ-statistic estimates departures from null (constant) diversification rates (dashed line).

orchids, (ii) volatile compounds from 34 non-orchid sources, and (iii) chemical preferences of 23 euglossine bee species. Our analyses revealed 585 volatile compounds that were acquired by euglossine bees. Of these, 54 (8%) were present in the floral scents of euglossine-pollinated orchids, and 59 (10%) were found in non-orchid fragrance sources (Fig. 3). Euglossine bees collect ~90 compounds classified as sesquiterpenes, but only 17 such compounds are produced by euglossine-pollinated Orchidaceae (fig. S15). Only 12 compounds collected by bees were found exclusively in orchid scents (Fig. 3). Thus, euglossine-pollinated orchids appear to provide only a small fraction of the compounds used by euglossines in courtship signaling. In fact, several chemicals commonly collected by male euglossine bees are also produced by a diverse array of tropical trees (*28*), fungi, and decaying vegetation (*25*). Thus, many of these compounds may have been abundantly available by the time Euglossini originated.

After euglossine-pollinated orchids originated and adapted to euglossine pollination via fragrance production, their diversification overlapped with that of several extant bee lineages (Fig. 1, A and B). To investigate whether the radiation of euglossine-pollinated orchids tracked the diversification of their bee pollinators, we used the γ-statistic of rate constancy to test for correlated deviations in diversification rates relative to a null model of constant lineage accumulation (*19*, *29*).

Euglossine bees exhibited a decrease in net diversification rates (origination minus extinction) toward the present, although a null hypothesis of constant diversification could not be rejected (Fig. 2B). In contrast, all three lineages of euglossine-pollinated Orchidaceae exhibited an increase in the diversification rates toward the present (Fig. 2B and table S1). Thus, despite the apparent overlap in their timing of diversification, lineage accumulation in these two groups appears to have been uncoupled, which is consistent with our conclusion that euglossine-pollinated orchids did not coevolve with their bee pollinators. This pattern may be explained by low extinction rates resulting from the recent origin of orchid lineages, or possibly by hybridization, which could bias coalescent times among lineages. Alternatively, recent shifts in pollinaria placement may have opened novel reproductively isolated niches for pollinator use, thus favoring unusually high speciation rates.

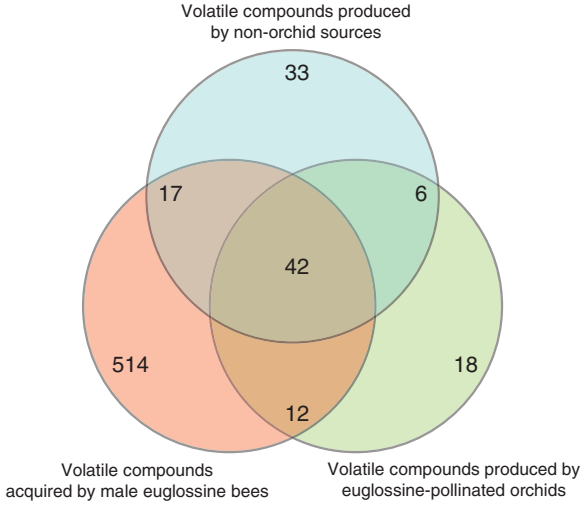

Fig. 3. Venn diagram showing chemical overlap among male-euglossine fragrance contents, euglossine-pollinated orchid floral scents, and volatile compounds produced by non-orchid fragrance sources (*19*).

To test whether our hypothesis of evolutionary asymmetrical dependencies was upheld in present-day ecological networks, we characterized the structure of bee-orchid interactions on the basis of bipartite networks of plant-pollinator associations (*19*). We found no evidence for reciprocally specialized bee-orchid associations (Fig. 1). Instead, the network architecture was highly nested (index = 5.1 to 9.1), where specialist lineages interact mainly with generalist partners. This pattern remained unchanged when orchid species were delimited on the basis of 1, 3, or 5% sequence

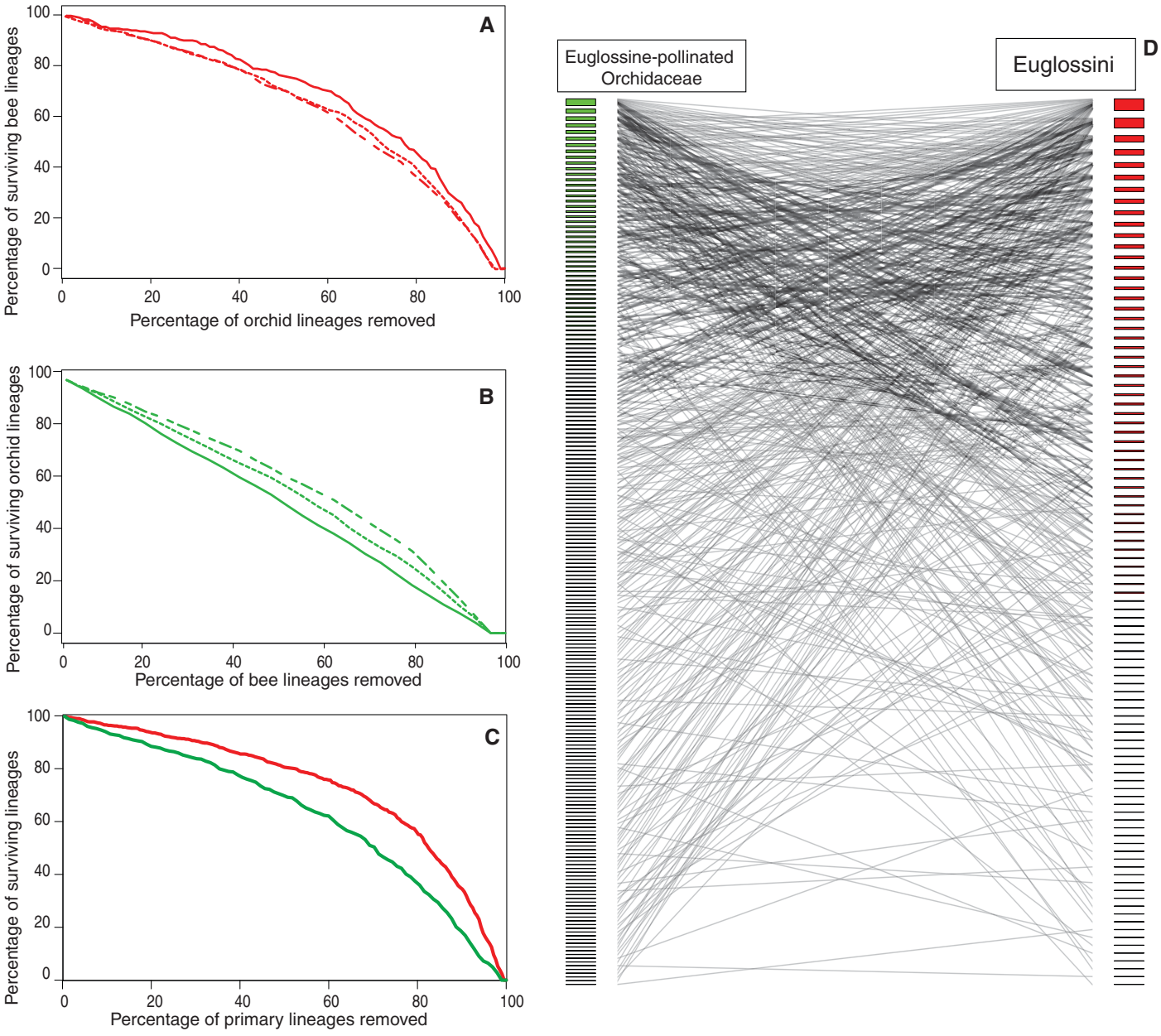

Fig. 4. Network architecture reveals asymmetrical dependence in the euglossine-orchid mutualism. (**A** and **B**) Simulated co-extinction of bees (A) and orchids (B) in response to random removal of their partners revealed faster declines of orchid lineages. Orchid species were delimited by 1% (solid), 3% (dotted), and 5% (double-dashed) pairwise sequence divergence cutoffs. (**C** and **D**) Simulated co-extinctions (C) performed on a larger data set (D) of 583 bee-orchid interactions (*19*) corroborated the asymmetrical dependency of this mutualism.

divergence cutoffs (fig. S13). To determine the degree of mutual dependency between orchid and bee lineages, we simulated co-extinction cascades by randomly removing one set of partners and recording the survival rate for the opposite partner (100 iterations). We found that the average decline rate of euglossine bees was relatively slow and nonlinear (Fig. 4A), whereas the decay of euglossine-pollinated orchids was rapid and almost linear (Fig. 4B). We corroborated this pattern by analyzing a larger data set of bee-orchid interactions derived from the literature, museum records, and field notes (Fig. 4, C and D) (*19*). This asymmetric dependency is associated with the lower degree (number of species interactions per lineage) of euglossine-pollinated orchids (average = 2.75) relative to that of euglossine bees (average = 5.94) and is consistent with plant-pollinator mutualisms forming networks with nested architectures (*7*, *8*). In fact, most mutualistic interactions exhibit highly nested architectures that distinguish them from nonmutualistic associations (*7*). Our results indicate that nestedness can evolve by one-sided evolution.

The co-radiation of flowering plants and insect pollinators has affected the evolution and diversification of terrestrial ecosystems. Our data do not support a scenario of coevolution between orchids and euglossine bees, but rather are consistent with the hypothesis that preexisting behaviors and/or sensory biases in insect lineages drove floral adaptation in specialized angiosperms (*30*). Moreover, our results shed light on the diversification of complex, free-living mutualistic associations, and demonstrate that radiations can result from interactions involving one-sided dependencies. The dependence of euglossine-pollinated orchids on their bee pollinators indicates that threats to euglossine bees and other insect pollinators may affect worldwide terrestrial ecosystems.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S15
Tables S1 to S6
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Single–Base Pair Unwinding and Asynchronous RNA Release by the Hepatitis C Virus NS3 Helicase

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Nonhexameric helicases use adenosine triphosphate (ATP) to unzip base pairs in double-stranded nucleic acids (dsNAs). Studies have suggested that these helicases unzip dsNAs in single–base pair increments, consuming one ATP molecule per base pair, but direct evidence for this mechanism is lacking. We used optical tweezers to follow the unwinding of double-stranded RNA by the hepatitis C virus NS3 helicase. Single–base pair steps by NS3 were observed, along with nascent nucleotide release that was asynchronous with base pair opening. Asynchronous release of nascent nucleotides rationalizes various observations of its dsNA unwinding and may be used to coordinate the translocation speed of NS3 along the RNA during viral replication.

Nonhexameric helicases belonging to superfamilies I and II are ubiquitous molecular motors essential for almost all aspects of nucleic acid metabolism (*1*, *2*). These enzymes use the free energy released in the hydrolysis of adenosine triphosphate (ATP) to generate the mechanical work needed to unzip base pairs in double-stranded nucleic acids (dsNAs). Structural (*3*–*6*), transient kinetic (*7*, *8*), and single-molecule fluorescence studies (*9*) of these proteins have suggested that these helicases unzip dsNAs in single–base pair (1-bp) increments, consuming one ATP per bp. However, there has been no direct evidence of 1-bp steps in the context of duplex unwinding, nor of the number of ATPs consumed per bp unzipped. The hepatitis C virus (HCV) encodes NS3, an RNA helicase that is essential for viral RNA replication (*10*) and particle assembly (*11*). NS3 is a superfamily II helicase (*12*, *13*) and shows structural resemblance to other nonhexameric helicase proteins (*14*). Although dimerization of NS3 is important for processive RNA helicase activity in vitro (*15*), several studies have shown that the NS3 monomer by itself has helicase activity (*16*–*19*). Thus, monomeric NS3 is a simple model system to understand the unwinding mechanisms of nonhexameric helicases in general.

Previous studies of NS3 that used optical tweezers revealed a cyclic movement of the helicase in discrete bursts of 11 bp, which at 50 μM ATP consists of smaller steps, 3.6 bp on average (*20*). Because the instrument resolution was 2 bp, it was not possible to directly observe 1-bp steps of the helicase. To measure the number of base pairs unzipped in each elementary cycle of the motor, we used a dual-trap optical tweezers instrument that can resolve angstrom-level displacement on a subsecond time scale (*21*, *22*) to follow the unwinding of a single RNA hairpin held between two optically trapped polystyrene beads (Fig. 1A). We operated the instrument without force feedback, holding the trap positions constant to reduce noise. Use of an RNA hairpin substrate with a homopolymeric G-C sequence (*16*) minimized the potential effect of sequence-dependent step sizes (*20*) and slowed the helicase unwinding speed (*16*, *23*, *24*), facilitating the detection of the elementary steps of the helicase.

After assembling a tether between two polystyrene beads, we flowed in NS3 together with ATP in buffer U (*19*). An NS3 concentration of 0.2 nM ensures that 98% of NS3 protein exists as a monomeric species in solution (*19*). When the RNA hairpin was held at an initial tension (15 to 21 pN) well below its unfolding force (*16*), NS3 bound spontaneously to a single-stranded RNA (ssRNA) loading sequence (10 bases) and unzipped the RNA hairpin. The increase in the end-to-end extension of the tether and the corresponding drop of the applied tension across the RNA molecule were recorded in real time. We used the worm-like chain (WLC) model (*25*) of RNA elasticity to convert the measured extension to the number of single-stranded nucleotides released by NS3, which indicates the number of base pairs opened at the hairpin junction.

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