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Escape from extreme specialization: passionflowers, bats and the sword-billed hummingbird

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A striking example of plant/pollinator trait matching is found between Andean species of *Passiflora* with 6–14-cm-long nectar tubes and the sword-billed hummingbird, *Ensifera ensifera*, with up to 11-cm-long bills. Because of the position of their anthers and stigmas, and self-incompatibility, these passionflower species depend on *E. ensifera* for pollination. Field observations show that the bird and plant distribution match completely and that scarcity of *Ensifera* results in reduced passionflower seed set. We here use nuclear and plastid DNA sequences to investigate how often and when these mutualisms evolved and under which conditions, if ever, they were lost. The phylogeny includes 26 (70%) of the 37 extremely long-tubed species, 13 (68%) of the 19 species with tubes too short for *Ensifera* and four of the seven bat-pollinated species for a total of 43 (69%) of all species in *Passiflora* supersection *Tacsonia* (plus 11 outgroups). We time-calibrated the phylogeny to infer the speed of any pollinator switching. Results show that *Tacsonia* is monophyletic and that its stem group dates to 10.7 Ma, matching the divergence at 11.6 Ma of *E. ensifera* from its short-billed sister species. Whether pollination by short-billed hummingbirds or by *Ensifera* is the ancestral condition cannot be securely inferred, but extremely long-tubed flowers exclusively pollinated by *Ensifera* evolved early during the radiation of the *Tacsonia* clade. There is also evidence of several losses of *Ensifera* dependence, involving shifts to bat pollination and shorter billed birds. Besides being extremely asymmetric—a single bird species coevolving with a speciose plant clade—the *Ensifera*/*Passiflora* system is a prime example of a specialized pollinator not driving plant speciation, but instead being the precondition for the maintenance of isolated populations (through reliable seed set) that then underwent allopatric speciation.

1. Introduction

Few evolutionary transitions in plant reproductive systems are irreversible, a conclusion now widely accepted based on changes in floral syndromes, sexual systems or self-pollination inferred on molecular phylogenies [1]. Among the exceptions may be the transition to hummingbird pollination. In a recent review of the topic of evolutionary reversibility, Barrett suggested that a directional bias in favour of transitions to, but not away from, pollination by hummingbirds may be due to the efficiency of these pollinators [2], the nature of genetic mutations in floral pigments that may make it difficult to return from red to blue or yellow colours [3] or the acquisition of thin long nectar tubes, difficult to modify [4]. Studies of floral trait change in the best-investigated North American systems, *Aquilegia* and *Penstemon sensu lato*, imply several shifts between moth, bee and hummingbird pollination, with a unidirectional trend towards long-tubed (hawkmoth- or hummingbird-pollinated) flowers in *Aquilegia* but not *Penstemon* [4,5]. This indicates trait reversibility over a few million years, the time frame for North American hummingbird/plant interactions [6]. But what about more extreme floral adaptations, such as those among many-centimetre-long flowers

pollinated by long-billed Andean hummingbirds? How long did it take for them to evolve, and is there a unidirectional trend from short flowers to long flowers as in the North American *Aquilegia* system?

To study these questions, we focused on the Passifloraceae, which are among the most species-rich groups with hummingbird-pollinated species. The largest genus is *Passiflora*, with 560 species of which 95% occur in tropical Central and South America, almost half (250 species) in subgenus *Passiflora* [7]. Within this subgenus, there is a group of species with floral tubes ranging from a few to 14 cm long (figure 1). These passionflowers are grouped into supersection *Tacsonia*, which comprises 62–64 species, all restricted to the high Andes at 1700 to approximately 4000 m ([8–11]; figure 2*b*). *Passiflora* supersection *Tacsonia* is characterized by several morphological traits, suggesting that the group might be monophyletic [9], although this has not really been tested. The best-sampled phylogeny so far included only seven *Tacsonia* species, which formed a clade [12]. While most species of the supersection have hummingbird-adapted flowers, the longest tubed-flowers are restricted to 37 species pollinated by the sword-billed hummingbird, *Ensifera ensifera*, whereas the 19 species with shorter tubed red flowers (hypanthium 1–3 cm long) are pollinated by shorter billed hummingbirds [13]. Bats pollinate another seven species that have greenish or white flowers [11,14]. Like most *Passiflora*, *Tacsonia* species are self-incompatible and depend on cross-pollination to set seed [10].

The long-tubed *Tacsonia* flowers exactly match the up to 11-cm-long bill of the sword-billed hummingbird (figure 3*a*), a common Andean species that occurs between 1400 and 4000 m.a.s.l. (figure 2*b* shows its geographical range). This bird species is the only pollinator capable of depositing pollen grains on the stigmas of these passionflowers while drinking nectar [15,16]. Northern *Ensifera* males have bills 10.4 cm long, females 11.2 cm long; birds from the southern part of the range have slightly shorter bills [17]. The morphological fit between the bird's bill length and the flower tubes and stamen and stigma positions, together with the overlap between *Ensifera* and the combined geographical ranges of the long-tubed passionflower species, make this relationship a clear case of plant/pollinator coevolution. Like many hummingbirds, *E. ensifera* is a trap-liner, regularly revisiting individual plants or flowers, which in the case of *Tacsonia* last 4–5 days. A dated hummingbird phylogeny shows that *E. ensifera* diverged from its short-billed sister species, *Pterophanes cyanopterus*, approximately 11.6 Ma [6].

Given that approximately 37 of the 62–64 species of *Tacsonia* are pollinated primarily or exclusively by the sword-billed hummingbird, while the remaining species are pollinated by short-billed hummingbirds or bats and assuming that the group is monophyletic, *Tacsonia* passionflowers make a suitable study system for addressing the question of specialization on, or de-specialization away from, a *single* pollinator species. Specialization on a single species entails the risk of interdependence, which may increase local or global extinction. Indeed, the scarcity of *E. ensifera* has been suggested as causing local extinction of *Passiflora mixta*, a member of supersection *Tacsonia*, in open landscapes in Ecuador [16]. Answering the question of increasing or decreasing specialization required a densely sampled phylogeny in which all pollination syndromes would be appropriately represented. Since we were interested in the evolutionary speed of any pollinator shifts, we applied a molecular clock model to the data to infer absolute divergence times for the *Tacsonia* passionflower clade.

2. Material and methods

(a) Plant material, DNA isolation, amplification and sequencing

The taxonomic names and authors, geographical origin, voucher information and place of deposition and GenBank accession numbers for all sequences produced for this study are listed in the electronic supplementary material, table S1. Approximately 0.2 g (dry weight) of leaf tissue was taken from 53 herbarium specimens of *Tacsonia*, representing 43 species from throughout the geographical and morphological range of the supersection. A total of 140 new sequences were deposited in GenBank. As outgroups, we used GenBank-downloaded sequences of 11 species from the *Passiflora* subgenera *Decaloba*, *Astrophea* and *Passiflora* (supersections *Coccinea* and *Passiflora*) based on Krosnick *et al*. [7]. As a more distant outgroup, we included *Paropsia madagascariensis* because its divergence time from other Passifloraceae has been estimated in another study [18] and could thus serve as a cross-validation point for our molecular clock dating (§2*b*).

DNA isolation relied on Nucleospin Plant II kits (Macherey-Nagel, Düren, Germany) and the manufacturer's protocol with the exception of incubation time, which was increased to 60 min. DNA concentrations were quantified using a NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific). The plastid *trn*L-F spacer region was amplified using the Taberlet *et al*. [19] primers c and f and an annealing temperature of 52°C. For samples that did not amplify with this primer combination, we additionally used the internal primers d and e. Our second plastid marker was the *ndhF* gene amplified with primers 5.5F and 10.2R of Davis *et al*. [20] with the same annealing temperature. As nuclear markers, we used the internal transcribed spacer (ITS) region amplified with the primer pair 5 and 4 of White *et al*. [21], and the low-copy glutamine synthetase gene (*ncpGS*) with the primer pair 687 and 994 of Emshwiller & Doyle [22]. PCR products were cleaned and purified, and then sequenced on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Warrington, UK). Chromatogram inspection and sequence assembly was done with CodonCode aligner (CodonCode Corporation), alignment with MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/), followed by visual inspection in Mesquite v. 2.75 [23]. All sequences were BLAST-searched in GenBank. Any ITS sequences with ambiguous base calls were removed from the final alignments to avoid using paralogous copies. For the *ncpGS* gene, which amplified in two distinct copies, one of a length of 545 and one of 657 aligned nucleotides, we only used the longer sequences.

(b) Phylogenetic analyses and molecular clock dating

Phylogenetic analyses used maximum likelihood (ML) as implemented in RAxML v. 7.6.3 [24] and Bayesian inference as implemented in BEAST v. 1.8.0 [25]. For the ML analysis, we used all four markers (*trnL-F*, *ndhF*, ITS and *ncpGS*), whereas for the Bayesian analysis, we excluded *ncpGS* because the long copy of this nuclear region amplified in only 17 species. Tree searches were carried out on the CIPRES science gateway portal [26]. In the absence of topological conflict (defined as greater than 75% ML bootstrap support) between the plastid and nuclear trees, data partitions were concatenated. To increase bootstrap support, we repeated the ML analyses with the full dataset of 44 species of *Tacsonia*, 10 outgroups, and 3581 aligned nucleotides and a reduced set of 37 species of *Tacsonia*, three

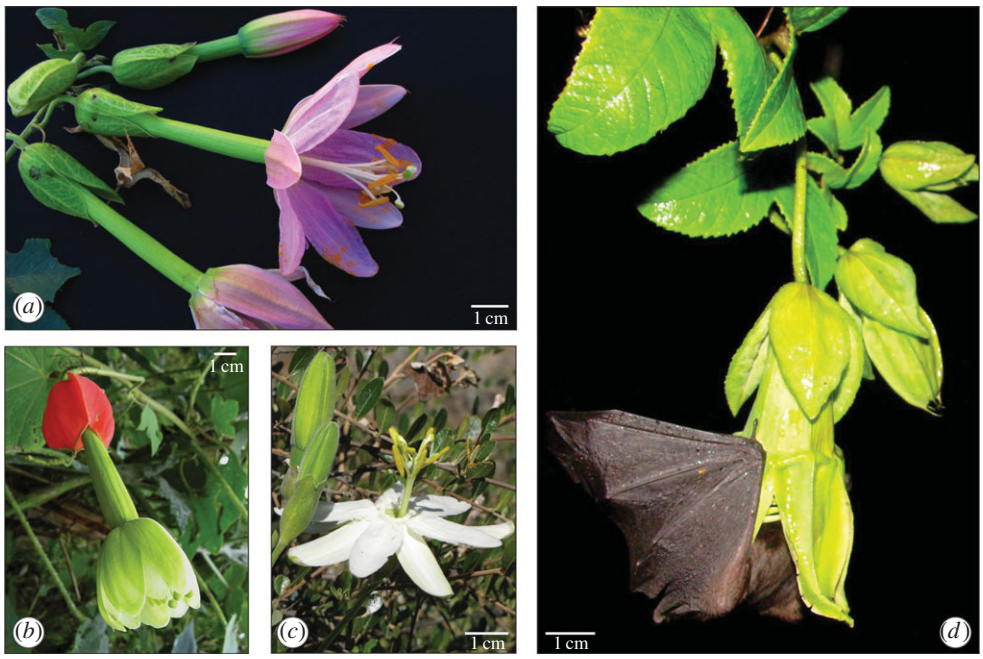

Figure 1. Representative species of *Passiflora* supersection *Tacsonia*. (*a*) *Passiflora tarminiana*, Peru, dependent on *E. ensifera* for pollination; (*b*) *Passiflora ampullacea*, Ecuador, dependent on *E. ensifera* for pollination. (*c*) *Passiflora peduncularis*, Peru, pollinated by bats. (*d*) *Passiflora unipetala*, Bellavista Cloud Forest Reserve, Pichincha, Ecuador, being visited by *Anoura fistulata*. Photo credits: figure (*a*) by P. M. Joergensen, (*b*) by G. Onore, (*c*) by T. Boza and (*d*) by N. Muchhala.

outgroups and 2867 aligned nucleotides. For the ML analyses, we used the GTR + G nucleotide substitution model (with four rate categories), but for the Bayesian runs, the slightly less parameter-rich HKY + G model (with four rate categories). To calibrate the genetic distances, we applied an ITS rate of 5.5×10^{-9} [27] to the ITS data matrix, without specifying a rate for the plastid data partition. We ran relaxed clock models using the uncorrelated lognormal model because the ucld.stdev value was more than 0.5, both with a Yule tree prior with gamma height distribution. MCMC chains were run for 40 million generations, sampling every 10 000th generation. Stationarity was checked in Tracer v. 1.5 [28], and output files were inspected in TreeAnnotator v. 1.8.0 (part of the BEAST package). The first 20% of trees were discarded as burn-in, and a posterior probability limit of 0.98 was set to retrieve a maximum clade credibility tree. All trees were viewed and annotated in FigTree v. 1.4.0 [29].

(c) Scoring of morphological flower traits and pollination syndromes

Morphological information for each species was taken from floras (cited in the electronic supplementary material, table S2), focusing on hypanthium length and flower colour. A character matrix was created in Mesquite, with hypanthium length divided into five categories (less than 1 cm, 1–2.9 cm, 3–5.9 cm, 6–9.99 cm and more than 10 cm). For each species, we searched for field observations on its pollinators, and species lacking direct pollinator observations were categorized based on flower colour and tube length, using the following criteria: (i) *E. ensifera*-dependent if flowers were pink, red or purple and hypanthium tubes more than 6 cm long; (ii) pollinated by other hummingbirds if flowers were pink, red or purple and tubes 1–5.9 cm long; (iii) pollinated by bats if flowers were greenish or white and/or there were actual observations of bat pollination; (iv) bee pollination, based on actual observations for some of the outgroup species (electronic supplementary material, table S2). Ancestral state reconstruction relied on maximum parsimony and likelihood optimization in Mesquite v. 2.75 [30], with the BEAST chronogram as the input tree and using the Mk-1 model of state transitions. As before, the tree was rooted on representatives of supersections *Coccinea* and *Passiflora*, based on Krosnick *et al*. [7].

3. Results

(a) Phylogeny and chronogram of *Passiflora* supersection *Tacsonia*

Because of variable success in PCR amplification for the nuclear and plastid regions, the individual alignments vary in the number of plant accessions (electronic supplementary material, table S1, shows all used sequences); especially the *ncpGS* alignments were highly incomplete, including only 17 sequences. The concatenated alignment comprised 44 *Tacsonia* species, 10 outgroups and 3581 nucleotides (electronic supplementary material, figure S1), but included many almost identical sequences. We therefore reduced the dataset to 37 *Tacsonia*, and just three outgrups (figure 2). The monophyly of *Tacsonia* is maximally supported (node 2 in figure 2), and there is statistical support for several nodes relevant to our study question, namely switches between *Ensifera* and short-billed hummingbirds. Switches between *Ensifera* and bats as pollinators occurred in the cloud forests of Bolivia, Peru and Ecuador, and one switch from short-billed hummingbirds to bats occurred in the group including *Passiflora trisecta*.

The molecular-clock chronogram is shown in figure 3, and a chronogram with 95% confidence intervals around the time estimates is shown as the electronic supplementary material, figure S2. The time tree contains slightly more outgroup species than figure 2 for the purpose of cross validation with results from a fossil-calibrated angiosperm-wide study [18]. In that study, the divergence between *P. madagascariensis* and *Passiflora* was dated to 28 (18–38) Ma, which is close to the 30.6 (20.2–40.4) Ma obtained in our study (electronic supplementary material, figure S2). The divergence of the *Tacsonia* clade from the most closely related *Passiflora* group occurred 10.7 (7.6–14.5) Ma, while the *Tacsonia* crown group dates to 8.4 (6.2–11.2) Ma.

In figure 3, 37 species of *Passiflora* supersection *Tacsonia* are coded as to their pollinators, with the basis for each coding shown in the electronic supplementary material, table S2

Figure 2. (*a*) Phylogeny for 37 species of *Passiflora* supersection *Tacsonia* based on 2867 aligned nucleotides of plastid and nuclear DNA markers. Species names in black refer to *Passiflora* species with nectar tubes 6–14 cm long and thus dependent on *E. ensifera* for pollination; names in red refer to species with nectar tubes less than 6 cm and pollinated by short-billed hummingbirds; in blue are species pollinated by bats; in green species pollinated by bees. Electronic supplementary material, table S2, provides the basis for each species' scoring. (*b*) Distribution map of *E. ensifera* from Birdlife International (2014) (www.birdlife.org). The distribution of supersection *Tacsonia* (in grey) completely matches that of *Ensifera* (in black). (Online version in colour.)

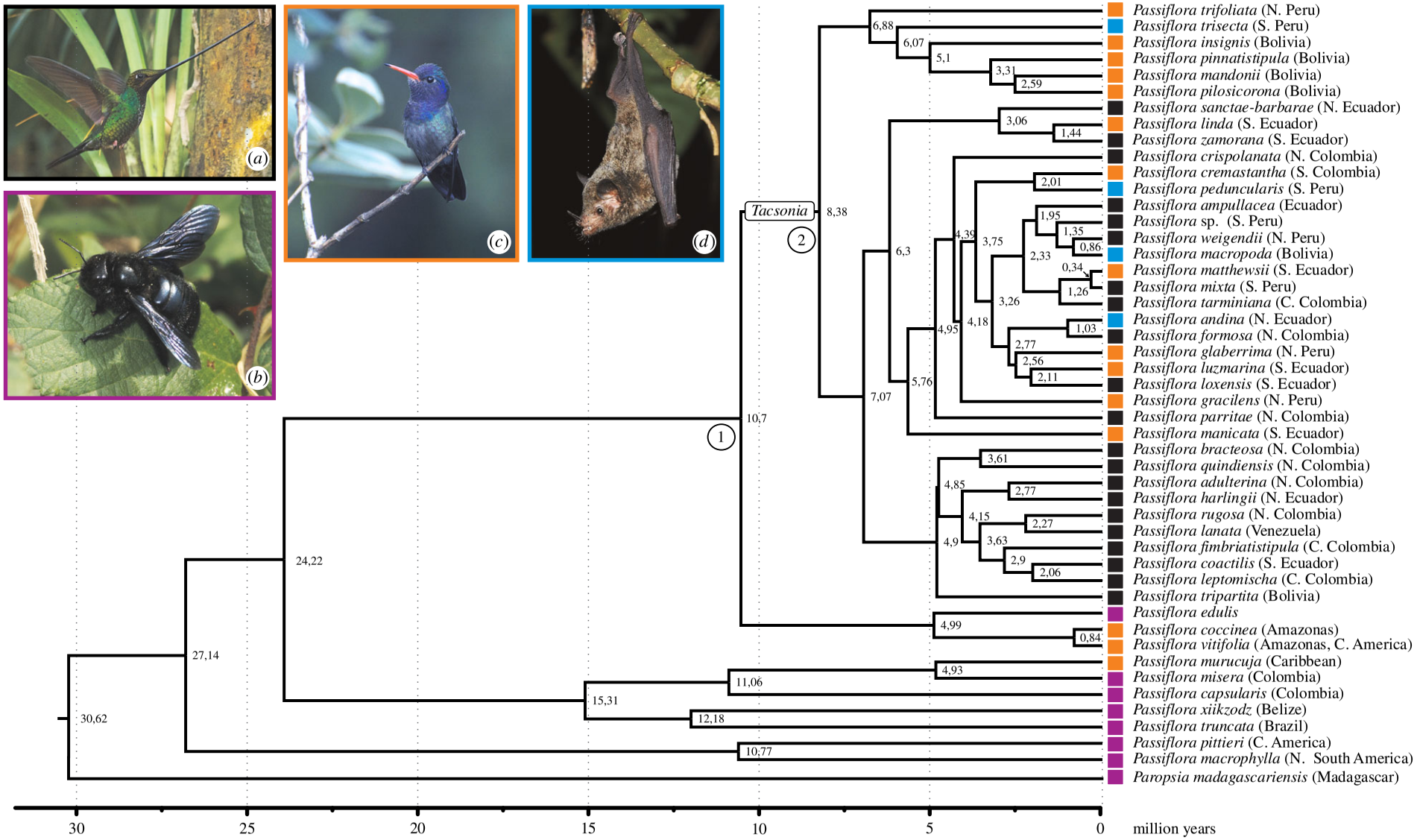

Figure 3. Molecular clock tree for 37 species of *Passiflora* supersection *Tacsonia*, with species colour-coded by pollinator type: bee, bat, short-billed hummingbird and *E. ensifera*. Electronic supplementary material, table S2, provides the basis for each species' scoring. The photos show (*a*) *E. ensifera*, (*b*) *Xylocopa* carpenter bee on a leaf of *Rubus*, (*c*) short-billed hummingbird (*Hylocharis cyanus*) and (*d*) the bat *Glossophaga commissarisi*, which pollinates species of *Tacsonia*. Electronic supplementary material, figure S2, shows the 95% confidence intervals around divergence times, figure S3 a parsimony-based ancestral state reconstruction and figure S4 a likelihood-based reconstruction of pollination syndromes. Photo credits: (*a*) J. C. Boone, (*b*) H. Mouret, (*c*) D. Sanchez and (*d*) K. Schneeberger (all from Wikipedia.org).

(*Material and methods*). Ancestral state reconstruction under parsimony (electronic supplementary material, figure S3) or ML (electronic supplementary material, figure S4) suggests that the ancestral state in *Tacsonia* may have been pollination by short-billed hummingbirds (node 2 in figure 3), followed by pollination by *Ensifera* at the next node; that node, however, lacks statistical support (see figure 2). A problem is that our species sampling in supersections *Coccinea* and *Passiflora*, the outgroups, is too poor to reliably infer their ancestral pollination by either bees or short-billed hummingbirds, which in turn prevents reliably inference at the root of section *Tacsonia*. It is nevertheless clear from our data that the adaptation to *Ensifera* evolved during the early radiation of the *Tacsonia* clade. Moves away from *Ensifera* to pollination by short-billed hummingbirds and bats occurred several times (figure 3), even when considering only statistically supported nodes (figure 2).

4. Discussion

Our data show that *Passiflora* supersection *Tacsonia* is monophyletic, diverged from the remaining *Passiflora* approximately 10.7 Ma, and underwent radiation at 9–8 Ma, matching a major uplift phase of the Northern Andes [31]. Our species sampling in the most closely related groups, supersections *Coccinea* and *Passiflora*, is too poor to reliably infer whether the ancestral pollination mode in *Tacsonia* was bee pollination or pollination by short-billed hummingbirds, but there is unambiguous support for an early coevolution between species of *Tacsonia* and *E. ensifera*, today the exclusive pollinator of over half the species (37 of 62–64). The interaction could have begun approximately 11 Ma, when *Ensifera* diverged from its relatively short-billed sister species, *P. cyanopterus* (bill length: 2.9 cm; [32]), an event dated to 11.6 Ma [6].

All species of *Tacsonia* are restricted to cloud forests between 1700 and approximately 4000 m altitude, the habitat of the sword-billed hummingbird, and the geographical distributions of the plant and animal partners in this mutualism overlap completely. The radiation of crown group *Tacsonia*, however, was mainly linked to the colonization of the newly arising Andean cloud forest habitat, not pollinator shifts because there are large clades that are entirely *Ensifera*-pollinated (figure 3; electronic supplementary material, figures S3 and S4). The *Tacsonia* mutualism with *Ensifera*, a reliable pollinator because of its trap-lining behaviour and strong flight ability, probably enabled populations to persist, that is, be pollinated and set seed, even in isolated patches, but was not *per se* the driving selective factor in species divergence (because there are too few switches to/from *Ensifera*-pollination).

Studying the effects of deforestation on the *P. mixta*/*E. ensifera* system at two sites in the Ecuadorean Andes, Lindberg & Olesen [16] found that few plants in the deforested, open land were visited by *E. ensifera* resulting in a low fruit set. This indicates the dependence of long-tubed passionflowers on their sole effective pollinator [16] and demonstrates the risks that dependence on a single pollinator species must carry, especially in plants unable to reproduce by self-pollination. Indeed, habitat fragmentation has been linked to the local extinction of at least four species of *Tacsonia* ([8]; M. Schwerdtfeger 2000, personal communication cited in [16]). This situation must have created the conditions conducive to shifts back to pollination by shorter billed hummingbirds or bats. Such shifts necessarily were linked to a shortening of nectar tube lengths since no other pollinator in the high Andes at 1700 to approximately 4000 m has the proboscis or bill length required to take up and deposit pollen from *Passiflora* with nectar tubes longer than 6 cm.

Bat pollination appears to have evolved both from short-billed hummingbird pollination and from *Ensifera*-pollination (figure 3; electronic supplementary material, figure S3 and S4) and to have resulted in shorter nectar tubes and whitish-greenish flowers (figure 1*d*). Our sampling includes four of seven bat-pollinated species and lacks the bat-pollinated *Passiflora colombiana*, *P. unipetala* and *P. weberbaueri*. Based on its morphology, *P. unipetala* is closest to the likewise bat-pollinated *P. andina* ([11]; figure 2); we do not know the relationships of *P. colombiana* and *P. weberbaueri*.

In *Aquilegia*, flower tube length evolved unidirectionally from short to long, with two types of transitions, bumblebee to hummingbird and hummingbird to hawkmoth [4]. In *Tacsonia*, there is no such irreversibility in tube length even though the colour switches from red bird-pollinated flowers to pale bat-pollinated flowers in *Tacsonia* resemble those in *Aquilegia*, which repeatedly switched from red bird-pollinated flowers to pale moth-pollinated flowers.

5. Conclusion

As shown here, the *Tacsonia* clade of *Passiflora* diverged from its sister group around 10.7 Ma and acquired long corolla tubes early during its evolution as a result of coevolution with the sword-billed hummingbird, itself dated to 11.6 Myr. Among the interesting features of this coevolution is its asymmetry, involving the interaction of one species of animal with a large clade of plants. Its specialized and therefore dependable pollinator enables even small and isolated population of *Tacsonia* to set seed, a situation conducive to allopatric speciation. Ongoing work in our laboratory is focusing on the few other plant species adapted to *Ensifera* and on understanding the variation in bill length from the northern to the southern part of the bird's range [17].

Data accessibility. All data were uploaded as the electronic supplementary material, tables S1 and S2, and all sequences have been submitted to GenBank. GenBank numbers are listed in electronic supplementary material, table S1.

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