LETTERS

Pollinator shifts drive increasingly long nectar spurs in columbine flowers

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Directional evolutionary trends have long garnered interest because they suggest that evolution can be predictable. However, the identification of the trends themselves and the underlying processes that may produce them have often been controversial[1]. In 1862, in explaining the exceptionally long nectar spur of *Angraecum sesquipedale*, Darwin proposed that a coevolutionary 'race' had driven the directional increase in length of a plant's spur and its pollinator's tongue[2]. Thus he predicted the existence of an exceptionally long-tongued moth. Though the discovery of *Xanthopan morgani* ssp. *praedicta* in 1903 with a tongue length of 22 cm validated Darwin's prediction[3], his 'race' model for the evolution of long-spurred flowers remains contentious[4]. Spurs may also evolve to exceptional lengths by way of pollinator shifts as plants adapt to a series of unrelated pollinators, each with a greater tongue length[5]. Here, using a species-level phylogeny of the columbine genus, *Aquilegia*, we show a significant evolutionary trend for increasing spur length during directional shifts to pollinators with longer tongues. In addition, we find evidence for 'punctuated' change in spur length during speciation events[6], suggesting that *Aquilegia* nectar spurs rapidly evolve to fit adaptive peaks predefined by pollinator morphology. These findings show that evolution may proceed in predictable pathways without reversals and that change may be concentrated during speciation.

The contemporary evolutionary 'fit' of nectar spurs and pollinator tongue lengths has been repeatedly demonstrated[4,7,8], suggesting that these traits have evolved owing to their interaction. However, there remains a controversy surrounding the mechanism by which this relationship evolves[4,5]. Under a hypothesis first proposed by Darwin[2] and later elaborated by Wallace[9], nectar spurs and pollinator tongues are engaged in a one-to-one coevolutionary 'race'. They suggested that, within a population, the plants with the longest nectar spurs have a selective advantage because their reproductive organs optimally contact pollinators and thus they achieve the greatest reproduction, whereas pollinators with the longest tongues have a selective advantage because they obtain the largest food reward (Fig. 1a). Spur length and pollinator tongue length then coevolve by following gradually shifting adaptive peaks (Fig. 1b). Alternatively, the pollinator shift hypothesis posits that tongue lengths are relatively fixed and spurs evolve in a one-sided process to fit them[5] (Fig. 1c). The tongue length of a pollinator may have evolved before an association with a plant species owing to selection on body size or in response to the spur lengths of other plant species[2]. When a plant becomes newly associated with a pollinator owing to dispersal to a new environment or changes in pollinator abundance, spurs then evolve to fit the pollinator's tongue length (Fig. 1c). Shifts to pollinators with markedly shorter tongues are predicted to be less
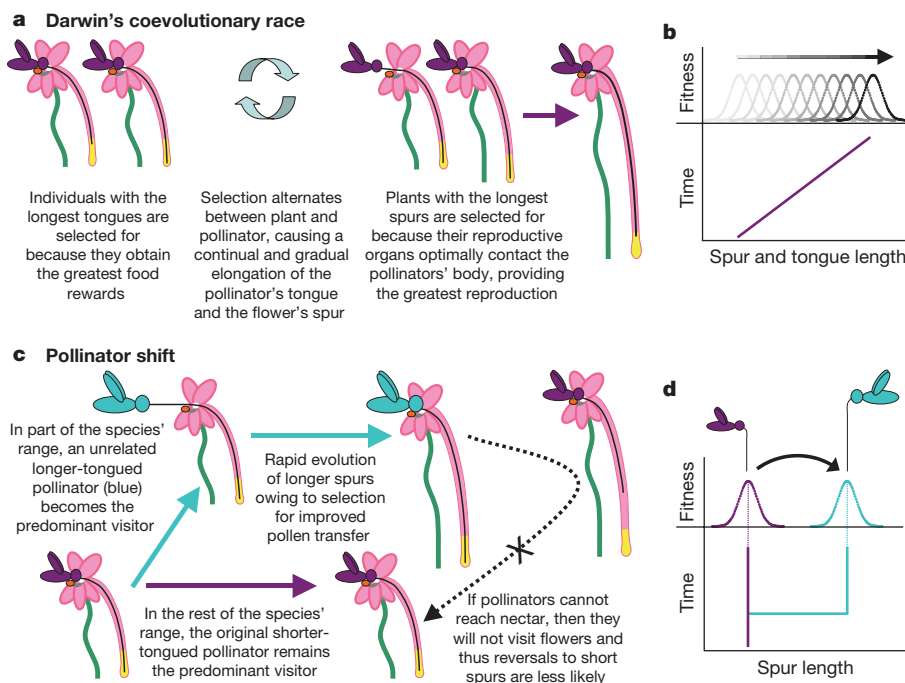

a Darwin's coevolutionary race

Individuals with the longest tongues are selected for because they obtain the greatest food rewards

Selection alternates between plant and pollinator, causing a continual and gradual elongation of the pollinator's tongue and the flower's spur

Plants with the longest spurs are selected for because their reproductive organs optimally contact the pollinators' body, providing the greatest reproduction

b Fitness / Time / Spur and tongue length

c Pollinator shift

In part of the species' range, an unrelated longer-tongued pollinator (blue) becomes the predominant visitor

Rapid evolution of longer spurs owing to selection for improved pollen transfer

In the rest of the species' range, the original shorter-tongued pollinator remains the predominant visitor

If pollinators cannot reach nectar, then they will not visit flowers and thus reversals to short spurs are less likely

d Fitness / Time / Spur length

Figure 1 | Two contrasting hypotheses for the evolution of exceptionally long nectar spurs. Darwin's coevolutionary race model (**a**, **b**), which posits a gradual increase in both the pollinator's tongue and the plant's nectar spur, and the pollinator shift model (**c**, **d**), where spur length evolves owing to a switch to a new pollinator with a longer tongue. These models differ in whether adaptive peaks are constantly increasing (**b**), or whether they are relatively fixed optima based on pollinators' pre-existing tongue lengths (**d**). They also differ in whether spur-length evolution occurs gradually (**b**) or in a punctuated fashion (**d**).

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likely because pollinators avoid flowers when they cannot obtain a reward[10,11]. Thus, this model also predicts that nectar spurs will generally become longer through time as spurs evolve to match a series of relatively stable adaptive peaks defined by tongue lengths (Fig. 1d).

A major difference between the two hypotheses is whether change occurs gradually within a species' lineage during a coevolutionary race with the same pollinator, or rapidly during shifts to new pollinators (Fig. 1). As shifts to new pollinators generally result in reproductive isolation[12], the change in spur length would be concentrated at speciation. Therefore, comparative phylogenetic analyses of the pattern of spur-length evolution can test both the common prediction that spurs generally become longer through time and also whether one of the hypotheses better explains the overall pattern of spur-length evolution.

The columbine genus *Aquilegia* (Ranunculaceae) is the result of a recent and rapid radiation[13], thought to be due to a key evolutionary innovation, namely nectar spurs[14]. To determine how nectar spur length evolved in columbines, we used a comparative phylogenetic analysis of all 25 North American taxa, which have nectar spurs that vary in length over a 16-fold range (7.5–123 mm). To provide the phylogenetic framework for comparative tests, we used a genomic survey with amplified fragment length polymorphisms (AFLPs) for all taxa (Supplementary Table 1). Bayesian analysis[15] of 1,576 variable markers for 176 individuals results in a highly resolved and well supported phylogeny for the North American *Aquilegia* clade (Supplementary Fig. 1a). Eighty per cent of the 30 interspecific nodes are resolved with greater than 95% posterior probabilities. The phylogenetic results are robust to several alternative methods of phylogenetic reconstruction (Supplementary Fig. 1b–d). To map
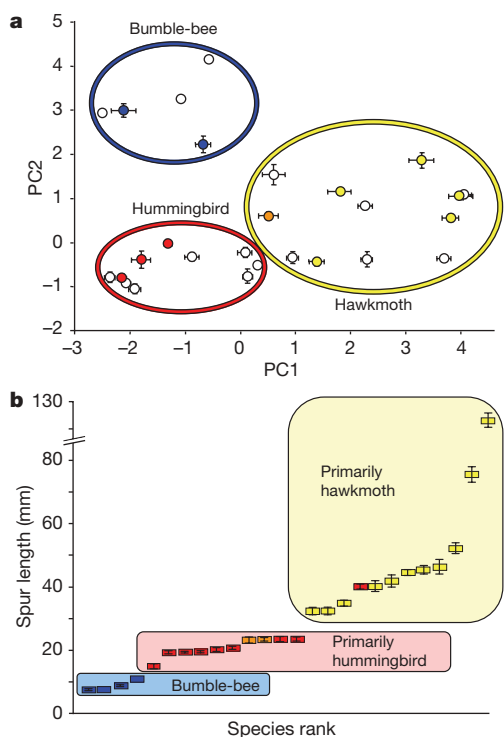
changes in pollination syndrome onto the phylogeny, we quantified multi-character pollination syndromes using principal components analysis (PCA) of ten floral traits, including nectar spur length. We found that the first two axes separate species into three distinct pollination syndromes (bumble-bee, hummingbird and hawkmoth) as described in ref. 16, and are consistent with direct pollinator observations for 11 of the taxa[17] (Fig. 2a; Supplementary Methods). These three syndromes have nearly non-overlapping distributions of spur lengths (Fig. 2b).

We used the phylogeny to determine the history of pollination syndrome evolution. Pollination syndromes are not all monophyletic (Shimodaira-Hasegawa test; $P < 0.00001$), indicating that multiple shifts have occurred. To identify the number of pollinator transitions, we mapped the three discrete pollination syndromes identified by PCA using local maximum-likelihood ancestral state reconstructions (Fig. 3a). This analysis indicates at least seven independent shifts between unrelated pollinators: two transitions from bumble-bee to hummingbird pollination and five shifts from hummingbird to hawkmoth pollination (Fig. 3a). To test for directionality in pollinator shifts, we developed a model describing the minimum number of transitions in pollination syndrome across the phylogeny[18]. Because transitions could be reversible, there are six possible transitions among the three syndromes. However, the maximum-likelihood solution resulted in only two significant transitions: bumble-bee to hummingbird, and hummingbird to hawkmoth (Fig. 3b; Supplementary Table 2). Therefore, there has been significant directionality in pollinator shifts and a lack of reversals in columbines.

Underlying the directionality of pollinator shifts are evolutionary transitions in spur length. To determine if spur length changes more when pollination syndromes shift, we used independent contrasts[19] and found that 73% of the total spur-length evolution occurs

Figure 2 | Quantification of pollination syndromes and the distribution of spur lengths in *Aquilegia*. a, Principal components analysis of 10 floral traits clusters species according to pollination syndrome, as defined in ref. 16. Species with published records of pollinator visitation are indicated with coloured symbols (blue, bumble-bee; red, hummingbird; yellow, hawkmoth; orange, hummingbird and hawkmoth; see Supplementary Methods). The first principal components axis (PC1) and the second (PC2) are plotted. Error bars, ±1 s.e.m. **b**, The distribution of spur lengths among the North American *Aquilegia* ranked by size. Taxa in each syndrome are colour-coded as in **a**. Error bars, ±1 s.e.m.
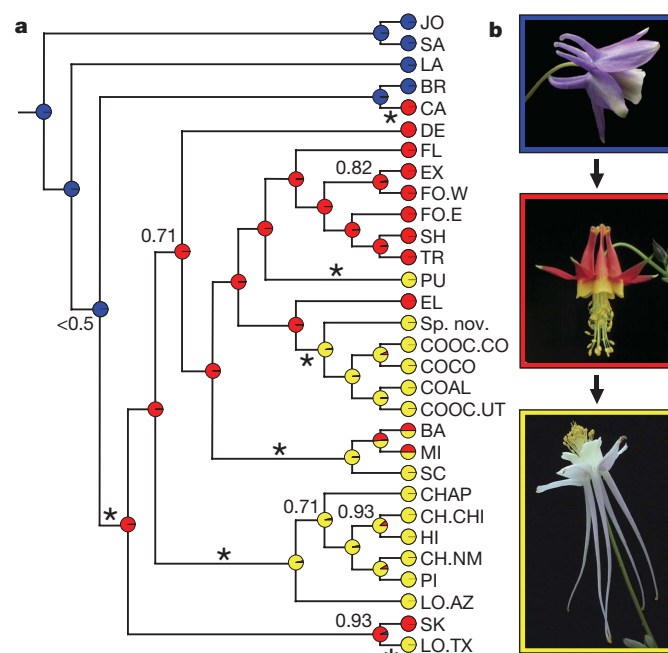

Figure 3 | Phylogenetic analysis of pollination syndrome evolution in *Aquilegia*. a, The majority-rule consensus bayesian cladogram with tips of the tree representing reciprocally monophyletic populations or species (see Supplementary Table 1). All interspecific nodes were supported by posterior probabilities >0.95 except where indicated. Species were assigned to pollination syndrome on the basis of Fig. 2a. The probability of each pollination syndrome occurring at ancestral nodes is indicated with pie charts at the nodes. Asterisks on the phylogeny indicate inferred shifts between pollination syndromes. **b**, Representative flowers of each pollination syndrome, with arrows indicating the only two significant transitions identified by the minimal model of evolution.

coincidently with pollinator shifts. This is significantly more than when pollinators do not change (Mann–Whitney, $z = 3.4$, one-tailed $P = 0.00016$). Though it is impossible to determine if spurs have lengthened or shortened when pollination syndromes remain constant, when syndromes shift they do so in ordered transitions (Fig. 3b) and thus we could infer the directionality of spur-length change. Twelve of the thirteen informative contrasts in pollination syndrome resulted in increases in spur length (sign test, one-tailed $P = 0.00171$). The only decrease in spur length occurred for the smallest pollination syndrome contrast (Fig. 4). Furthermore, larger pollination syndrome contrasts are correlated with greater spur-length evolution (regression analysis, $r^2 = 0.669$, $F = 56.58$, d.f. = 28, $P = 1.71 \times 10^{-8}$; Fig. 4). These finding are robust to the alternative pollination syndrome assignments for *Aquilegia micrantha* and *Aquilegia barnebyi* (Supplementary Methods) and several alternative branch length transformations (Supplementary Table 3).

The pollinator shift model also predicts that changes in pollination syndrome, and thus spur length, will occur in a 'punctuated' fashion[6], primarily during speciation events where at each node, one daughter lineage rapidly evolves to occupy a new adaptive peak and the other retains the ancestral condition (Fig. 1d). We tested for the existence of one or more stable evolutionary states in spur length by comparing models that incorporate 'adaptive peaks'[20] to a null model of brownian motion. We found significant preference for a model with three distinct optima (likelihood ratio 33.03, d.f. = 6, $P = 1.8 \times 10^{-6}$; Table 1), indicating that once a lineage has adapted to one pollination syndrome, spur length remains relatively stable until there is a transition to another pollination syndrome. We also explicitly tested whether spur length evolves gradually, in proportion to branch length, or in a punctuated fashion at speciation[6] (Fig. 1d), and found that the punctuated model was preferred (Supplementary Discussion; Supplementary Table 4).

Spur-length evolution within a pollination syndrome may often be due to Darwin's coevolutionary race hypothesis, though the pollinator shift model may also explain some of this variation. For example, pollinator shifts between distantly related hawkmoths may account for the substantial variation in spur length among hawkmoth-pollinated *Aquilegia* species (Fig. 2b). Hawkmoth pollinators with relatively short tongues, for example, *Hyles lineata* and *Eumorpha achemon* (38–46 mm)[21], belong to the Macroglossinae, whereas those with long tongues, for example, *Sphinx vashti* and *Manduca* spp. (54–137 mm)[21], belong to the Sphinginae. These subfamilies have been separated for at least 13.3 Myr (ref. 22), far longer than the age of the

Table 1 | Alternative models of spur-length evolution

Evaluation method	BM model	Number of optima for adaptive models	
		One	Three
LnL	47.93	47.94	14.90*
AIC	51.93	55.94	26.90†
SIC	54.73	61.54	35.31†

Spur-length evolution was modelled using a non-adaptive, brownian motion (BM) model, as well as adaptive models constrained around one or three adaptive optima[20]. Alternative models were evaluated by log likelihood (LnL), Akaike information criterion (AIC) and Schwarz information criterion (SIC). As statistical criteria for model selection, AIC uses the LnL and the number of parameters whereas the SIC additionally incorporates the number of observations. In both methods, lower values are preferred.
* $P = 1.8 \times 10^{-6}$.
† Strongly preferred[20].

entire North American *Aquilegia* clade[23]. Thus, tongue-length differences among hawkmoths were probably established before their association with *Aquilegia,* perhaps owing to coevolutionary races with other plant species. Therefore, transitions within pollination syndromes may also be consistent with the one-sided pollinator shift model.

Our finding that spur length in *Aquilegia* evolves largely through directional transitions among pollination syndromes suggests that reaching some adaptive optima may require intermediate 'stepping stones'. Thus, the lack of an intermediate adaptive peak may prevent a species from shifting to an even more extreme morphology. For example, hummingbirds are absent from Eurasia, but hawkmoths do exist there[21]. Significantly, there is very little variation in spur length among Eurasian *Aquilegia* species (4.0–21.5 mm; mean ± s.e.m., 13.0 ± 0.59 mm) and no clear examples of the hawkmoth pollination syndrome. The length of the longest spurred Eurasian *Aquilegia* species (*Aquilegia alpina*, 21.5 mm) is more than 10 mm shorter than the shortest North American hawkmoth-pollinated species (*Aquilegia pubescens*, 32.4 mm). In the absence of hummingbirds or another appropriate pollinator with a comparable tongue length, Eurasian *Aquilegia* species may lack an adaptive 'stepping stone' necessary to reach the hawkmoth adaptive optimum.

Although Darwin's coevolutionary race may be responsible for spur-length evolution within species, our comparative phylogenetic evidence indicates that the majority of spur-length evolution in columbines fits the pollinator shift model. Because columbines have experienced a recent and rapid adaptive radiation[13], it is likely that pollinator tongue lengths were predominantly established before spur-length evolution, which has thus evolved primarily during repeated and directional shifts among pollination syndromes. Of particular note is the finding that shifts in pollination syndrome have occurred without reversals, resulting in the progressive lengthening of nectar spurs. Our results also indicate that large changes in spur length occur disproportionately at speciation events, resulting in 'punctuated' morphological changes.

METHODS SUMMARY

We sampled 155 individuals of all 25 North American *Aquilegia* taxa, and 21 individuals of 8 outgroup taxa, and genotyped them for AFLP markers. We conducted a bayesian phylogenetic analysis of the binary data under a restriction-site model in MRBAYES[15] and compared the resulting phylogeny with parsimony and distance-based phylogenetic analyses (Supplementary Methods). The majority rule consensus tree from the bayesian analysis was pruned to populations or species for subsequent comparative analyses.

For 10 floral traits, we measured 10–52 individuals representing 1 to 5 populations per species for 23 of the 25 taxa in an among-species principal components analysis (see Supplementary Table 5 for axis loadings). Trait data for the remaining two species were estimated from the literature (Supplementary Methods). We conducted local maximum-likelihood ancestral state reconstructions, and tested for directionality of pollination syndrome evolution using Bayes Multistate[18]. Adaptive models of spur-length evolution were compared using the likelihood ratio test, the Akaike information criterion (AIC) and the Schwarz information criterion (SIC) in OUCH![20], and the punctuated-speciational versus gradual models were compared using AIC values in CoMET[6].
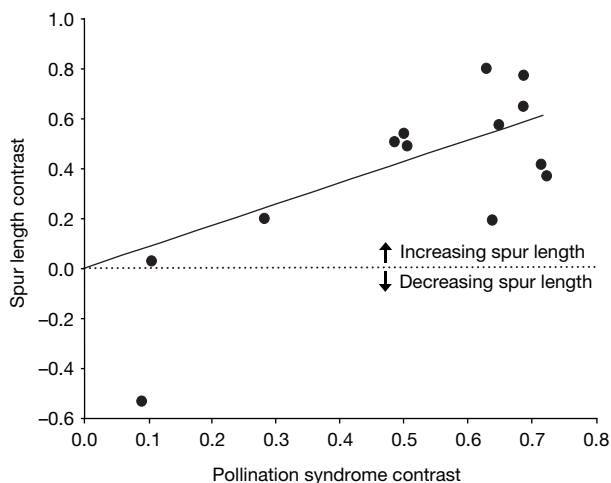

Figure 4 | Independent-contrasts regression analysis of pollination syndrome and spur-length evolution. Pollination syndromes were ordered so that contrasts reflected the minimum model's structured transitions (Fig. 3b). Transitions in pollination syndrome are significantly correlated with increasing spur lengths.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J.B.W. (jbwhittall@ucdavis.edu) or S.A.H. (hodges@lifesci.ucsb.edu).

METHODS

Taxonomic sampling. One to three populations (2–5 individuals per population) of all 25 North American *Aquilegia* taxa were sampled (Supplementary Table 1). Outgroups consisted of seven Eurasian *Aquilegia* species and *Semiaquilegia adoxoides*.

AFLP methodology. DNA was extracted from fresh leaf material of wild collected plants or plants grown from wild collected seeds. The AFLP protocol followed a modified version of that in ref. 24, with a $5\times$ digestion with *Eco*RI and *Mse*I and two fluorescently labelled E-primers in each selective amplification. Products from 20 selective amplifications were separated on a LiCor 4200 DNA sequencer and variable bands were manually scored with SAGA MX (LiCor).

Phylogenetic analyses. A bayesian analysis of the binary data incorporating differential rates of band gains and losses was conducted with the RESTRICTION model in MRBAYES v3.0 beta 4[15]. As only variable AFLP bands were scored, CODING was set to VARIABLE. Three runs of five million generations were sampled every 100 generations then combined after removing the burn-in (1.5 million generations per run). Posterior probabilities and branch lengths were calculated from the consensus of the posterior distribution of trees. To determine the robustness of the phylogenetic estimate, we conducted a series of additional phylogenetic analyses under distance and parsimony optimality criteria (see Supplementary Methods). For comparative analyses, the bayesian phylogeny was pruned to populations or species using the average branch-length to the clade's descendants and outgroups were removed. The rooted phylogeny was then converted to an ultrametric tree using the NPRS algorithm in r8s (ref. 25) with a smoothing factor of -0.00001. The single unresolved node in the bayesian consensus phylogeny ($PP < 0.5$) was grafted in both alternative arrangements with a minimum branch length (1×10^{-6}) for comparative analyses that require a strictly bifurcating phylogeny[26].

Quantification of floral traits. Ten floral traits were quantified for all species (see Supplementary Methods). PCA among species was then used to validate previously assigned pollination syndromes[16,17] and to help assign syndromes to the few remaining species (see Supplementary Methods). Trait loadings on the first two principal components axes are presented in Supplementary Table 5. *Aquilegia micrantha* and *A. barnebyi* were considered polymorphic for hummingbird and hawkmoth pollination syndrome or, when polymorphic coding was not an option, separate comparative analyses were conducted with these species coded as alternative character states.

Spur lengths. Spur lengths were log-transformed (Kolmogorov–Smirnov test for normality, $P > 0.15$) for comparative analyses.

Pollination syndrome evolution. Local maximum-likelihood ancestral state reconstructions for the three discrete pollination syndromes were conducted in Bayes Multistate[18]. This program was then used to estimate a minimum model of pollination syndrome evolution (Supplementary Table 2). We first tested for irreversible transitions by restricting the smallest rates to equal zero. Likelihood ratios >2 were used to determine if rates were significantly different from zero. To determine if any of the remaining transition rates were significantly different from one another, we compared a two-rate model with a one-rate model. We used a likelihood ratio test to determine statistical significance following a χ^2 distribution with one degree of freedom based on the difference in the number of free parameters between the two models.

Calculation of independent contrasts. We tested for correlated evolution of pollination syndrome and spur length using independent contrasts[19] as implemented in the PDAP module[27] of Mesquite[28]. Pollination syndromes were coded as discrete states as assigned (Fig. 2a) and ordered on the basis of the minimum model of pollination syndrome evolution (Fig. 3b). Combining continuous and discrete characters does not violate the statistical assumptions of independent contrasts[29]. Independent contrasts analysis can be sensitive to branch-length assignments, so we used a branch-length diagnostic[29] and several alternative branch-length transformations (Supplementary Table 3). All contrasts, including non-informative pollinator contrasts, were included in the regression analysis providing a conservative estimate of correlated evolution between pollination syndrome and nectar spur length[29].

Tempo and mode of spur-length evolution. We tested a series of alternative evolutionary models to determine the tempo and mode of spur-length evolution. First, we examined the fit of several adaptive models in OUCH![20] to compare the maximum likelihood of the brownian motion model with successively more complex adaptive models that incorporate an Orenstein-Uhlenbeck elasticity parameter modelling adaptive optima. We compared adaptive models with one versus three optima as described in ref. 20. The fit of the data to the models was estimated using a likelihood ratio test, the Akaike information criterion and the Schwarz information criterion.

We examined the importance of topology and alternative branch-length scaling using CoMET[6] implemented in Mesquite[28]. We compared results from a gradual model of evolution (termed pure-phylogenetic distance[6]) to a punctuated, speciational model (termed punctuated average equal[6]). Alternative models were evaluated using the Akaike information criterion. The maximum likelihood solution assigns which descendent lineage evolves and which remains constant.

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