Coevolutionary elaboration of pollination-related traits in an alpine ginger (*Roscoea purpurea*) and a tabanid fly in the Nepalese Himalayas

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Summary

- Geographical variation in the interacting traits of plant–pollinator mutualism can lead to local adaptive differentiation. We tested Darwin's hypothesis of reciprocal selection as a key driving force for the evolution of floral traits of an alpine ginger (*Roscoea purpurea*) and proboscis length of a tabanid fly (*Philoliche longirostris*).
- We documented the pattern of trait variation in *R. purpurea* and *P. longirostris* across five populations. At each site, we quantified pollinator-mediated selection on floral display area, inflorescence height and corolla length of *R. purpurea* by comparing selection gradients for flowers exposed to natural pollination and to supplemental hand pollination. Reciprocal selection between plant and fly was examined at two sites via the relationship between proboscis length and nectar consumption (fly benefit) and corolla length and pollen deposition (plant benefit).
- Local corolla tube length was correlated with local fly proboscis length among the five sites. We found strong linear selection imposed by pollinators on corolla tube length at all sites, but there was no consistent relationship of fitness to inflorescence height or floral display area. Selection between corolla length and proboscis length was reciprocal at the two experimental sites examined.
- The geographical pattern of trait variation and the evidence of selection is consistent with a mosaic of local, species-specific reciprocal selection acting as the major driving force for the evolution of corolla length of *R. purpurea* and proboscis length of *P. longirostris*.

Introduction

Pollinator-mediated selection is one of the key evolutionary forces underlying the remarkable diversity of angiosperm flowers (Darwin, 1862; Nilsson, 1988; Fenster *et al.*, 2004). Selective pressure imposed by the most frequent and most effective visitors may act on advertising traits, such as size, colour, fragrance and inflorescence height, and on mechanical traits, such as corolla tube length (Stebbins, 1970; Anderson *et al.*, 2014; Newman *et al.*, 2015). In turn, floral traits may impose selection on pollinator traits, leading to reciprocal coevolution (Muchhala & Thomson, 2009; Pauw *et al.*, 2009).

Ever since Darwin's (1862) prediction that the long nectar spur of the Malagasy star orchid (*Angraecum sesquipedale*) was the coevolutionary outcome of reciprocal selection with a long

proboscid hawkmoth, the relationship between corolla tube length and pollinator proboscis length has been a model for investigating coevolution (Zhang *et al.*, 2013). According to Darwin's hypothesis, selection on the pollinator favours longer proboscides to achieve easy access to the nectar, while selection on the plant favours longer corolla tubes, which ensure the pollinator's contact with the reproductive parts and thus maximize pollen transfer (Darwin, 1862; Muchhala & Thomson, 2009). This kind of selective regime can lead to reciprocal coevolution that escalates the length of both traits (Benkman *et al.*, 2003; Anderson & Johnson, 2008; Thompson, 2013). However, most studies on Darwin's hypothesis have focused mainly on the evolution of tube length (Nilsson, 1988; Maad, 2000; Alexandersson & Johnson, 2002), while there is much less evidence of reciprocal coevolution (Muchhala & Thomson, 2009; Pauw *et al.*, 2009; Thompson *et al.*, 2013).

Moreover, the geographical mosaic theory of coevolution holds that selective dynamics may vary considerably in space and time, leading to coevolutionary hotspots, where selection is reciprocal, and coldspots, where selection is one-sided or absent (Gomulkiewicz *et al.*, 2000; Thompson & Cunningham, 2002; Thompson, 2005). Such dynamics may yield geographical variation in the degree of coevolution observed in populations across a landscape (Steiner & Whitehead, 1990; Anderson & Johnson, 2008), a bias in trait matching in favour of one species (Benkman *et al.*, 2003; Anderson & Johnson, 2008; Pauw *et al.*, 2009; Toju, 2011), or bimodal distributions of traits within a population (Wasserthal, 1997; Anderson *et al.*, 2009; Pauw *et al.*, 2009; Zhang *et al.*, 2013). Variation in floral traits and pollinator proboscis length may be independently evolved responses to underlying changes in the physical environment, as reflected in latitude and altitude (Anderson & Johnson, 2008; Newman *et al.*, 2014), or the consequence of allometric scaling of the traits with overall size (plant size, flower size, or pollinator's body size) (Anderson & Johnson, 2008). These alternative sources of geographical patterns in the traits of interest must be considered in any examination of coevolution (Thompson, 2013).
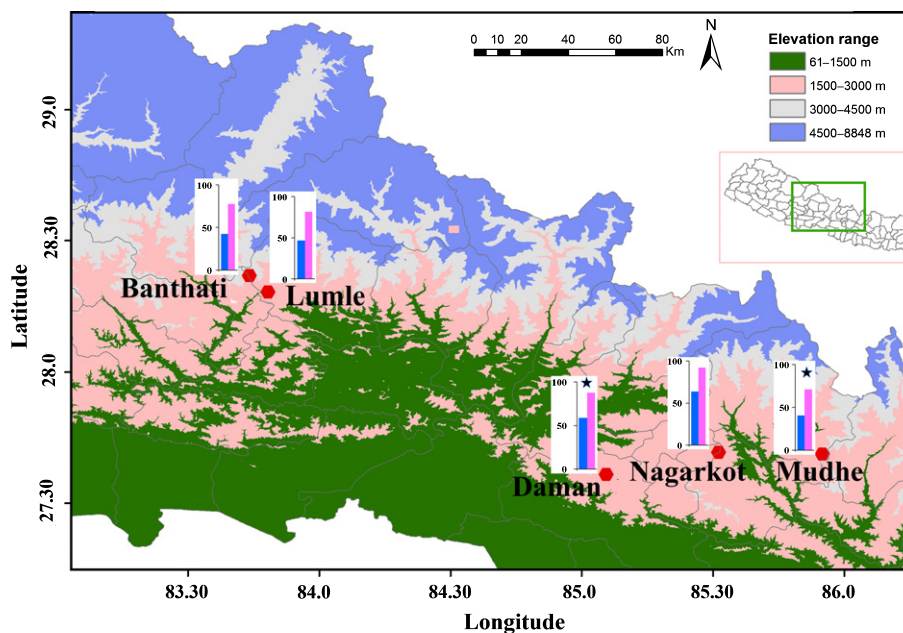
We examined the interaction between an alpine ginger, *Roscoea purpurea* (Zingiberaceae), and its obligate pollen vector, *Philoliche longirostris* (Diptera: Tabanidae) (Paudel *et al.*, 2015). Our preliminary field observations indicated that inflorescence height, floral display area, and corolla tube length of *R. purpurea*, as well as proboscis length of *P. longirostris*, varied across the wide range of habitats in which the populations occur. Moreover, the variation in floral length and fly proboscis length appeared to be concomitant, in a manner consistent with coevolution (Paudel *et al.*, 2015).

We examined the apparent coevolution of plant and pollinator traits in five populations in the Nepalese Himalayas. (1) If local coevolution has occurred, the lengths of floral tubes and fly proboscides should be correlated across sites. Such correlation is consistent with but does not by itself demonstrate coevolution. (2) We tested whether pollinator-mediated selection is acting on plant and floral traits in each population, one side of the possible reciprocal selection. Our test compares phenotypic selection gradients under natural pollination with the corresponding gradients obtained when flowers are given supplemental pollination by hand, which supplies a surfeit of pollen and obviates the effect of pollinator visitation on pollen delivery. (3) If reciprocal selection is occurring, the foraging success of flies should be positively associated with their proboscis length, and more specifically with the match between proboscis length and the length of the corolla tube they probe. In turn, plants should receive more pollen from visitors with shorter proboscides. Reciprocal selection is more difficult to measure and we tested this in only two of the five populations. These tests do, however, provide a direct look at the processes that putatively occur at all sites. (4) Finally, we examined whether the floral and pollinator traits are varying in response to third factors, related either to the overall body size of the plant or the fly or to multiple unknown factors summarized by geographical and altitudinal differences among sites.

Materials and Methods

Study sites

The research was conducted at five widely separated sites in Nepal (Fig. 1). Geographical details of the study areas are

Fig. 1 Study sites and geographical covariation in corolla tube length of *Roscoea purpurea* and proboscis length of *Philoliche longirostris* across five populations in the Nepal Himalayas. Red hexagons represent the study sites. Pink bars, corolla tube length (mm) of *R. purpurea*; blue bars, the proboscis length (mm) of *P. longirostris*. Names below the red hexagons represent the names of study sites. Asterisks indicate the sites of the reciprocal selection experiment.

presented in Supporting Information Table S1. These sites are located at different altitudes within the Nepalese range of *Roscoea purpurea* Sm. and *Philoliche longirostris* Hardwicke. At all sites, *R. purpurea* was the most abundant species in flower during our observation period and *P. longirostris* was observed to be its exclusive floral visitor (Paudel *et al.*, 2015). Each site covers *c.* 0.5–1 km² with > 5000 flowering individuals, so that a fly had sufficient opportunity to interact with flowers within a site. However, movement of flies among the sites is unlikely because all study sites are at least 20 km apart and separated by deep valleys with unsuitable habitat for the flies.
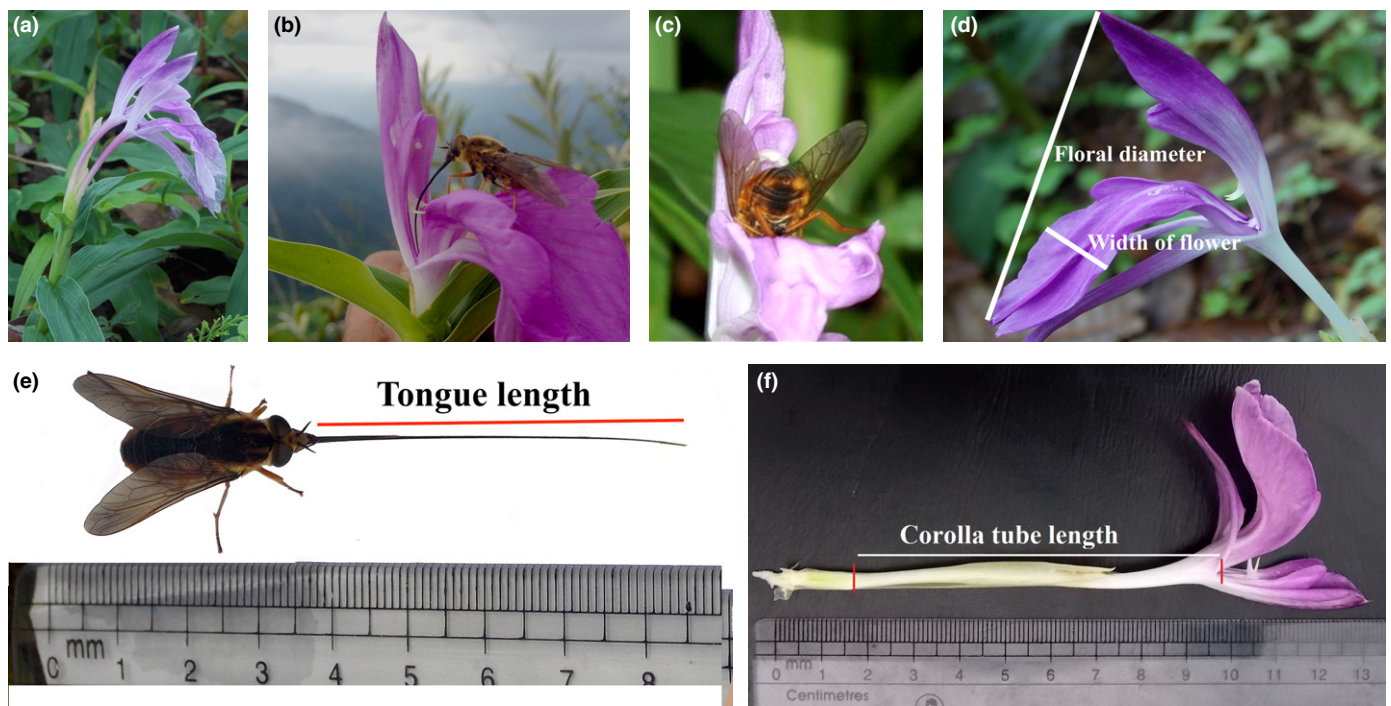
Study species

Roscoea purpurea is a Himalayan endemic perennial herb found growing in *Pinus* and *Rhododendron* forests on subalpine to alpine slopes between 1520 and 3100 m above sea level (Cowley, 2007). Plants are usually 20–60 cm tall, with an annual leafy shoot produced from erect rhizomes. Flowering occurs in the summer, from June to September. Flowers are usually purple and have an elongated corolla tube with nectar (Cowley, 2007). Previous bagging experiments showed that *R. purpurea* is self-compatible but not autogamous or apomictic, and thus requires floral visitation for reproductive success. Pollination in *R. purpurea* occurs via an obligate pollen vector, *P. longirostris*

(Paudel *et al.*, 2015). *Philoliche longirostris* is the only species of long-tongued fly distributed in the Himalayas and has the longest proboscis among all members of the Tabanidae (Goldblatt & Manning, 2000). The seasonal prevalence of this fly synchronizes closely with the peak blooming period of *R. purpurea* (Sen, 1931; Paudel *et al.*, 2015). Pollen transfer occurs when a fly pushes against the staminal appendages that extend from the base of the stamen at the entrance of the corolla tube. This action causes the anther and the style and stigma to descend and touch the fly's back (Paudel *et al.*, 2015).

Geographical covariation of interacting traits

We explored the pattern of geographical covariation between plant traits and fly traits across the five study populations. At each site, we randomly selected 40 flowering individuals and measured plant height, floral dimensions, and corolla tube length (Fig. 2) to the nearest millimetre. We also captured 20 individuals of *P. longirostris* with a net and measured body length, thorax breadth, and proboscis length with digital calipers. Differences among populations in corolla tube lengths and fly proboscis lengths were analysed by one-way ANOVA. Regression of mean floral tube length against mean proboscis length for each site was used to explore the geographical covariation between the two traits. A positive association is the predicted consequence of

Fig. 2 (a) Flowering individual of *Roscoea purpurea*. (b, c) Reconstruction of the interaction between *Philoliche longirostris* and *R. purpurea* using captured flies. (b) In a flower with a corolla tube shorter than the proboscis length of a visiting fly, the fly is not forced to probe deeply inside the flower and thus the fly can easily drain the nectar. In this situation, the fly does not touch the anther and stigma and thus does not provide a pollination service to the plant. (c) Flowers with a longer corolla tube force the fly to push its head into the corolla tube to obtain nectar. This action triggers a lever-like mechanism via staminal appendages, and the anther–stigma apparatus bends over the body of the fly. This eventually ensures pollination. (d) Measurements of floral display dimensions in *R. purpurea*. (e) Proboscis length measurement for *P. longirostris*. (f) Corolla tube length measurement in *R. purpurea*.

reciprocal selection and therefore necessary evidence to demonstrate a geographical mosaic of coevolution, although it would be insufficient evidence in itself.

Any geographical pattern in corolla tube length or proboscis length could be the consequence of underlying changes in plant size, flower size, or insect body size, or a consequence of biotic or abiotic factors that differed with differences in latitude, longitude and altitude among the sites. Hence, multiple regression was used to examine possible allometric relationships involving corolla tube length and pollinator proboscis length. Plant height and flower size were taken as predictive variables for corolla tube length, while body length and breadth of fly were considered as predictors for fly proboscis length. For these analyses, we assumed that traits vary independently across populations. However, if the variation is structured by gene flow or common descendent, populations with geographical proximity will have similar trait values (Anderson & Johnson, 2008; Newman *et al.*, 2014). We tested this possibility using Mantel tests with 9999 permutations in the *ade4* package of R (v.3.0.2). Nonsignificant or negative relationships between geographical or altitudinal distance and trait variation signify that trait values are the outcome of independent evolution at local levels rather than of common descendent or gene flow across sites (Anderson & Johnson, 2008; Newman *et al.*, 2014).

Selection on floral traits

We considered fruit set and seed production as proxy measures of fitness of *R. purpurea*, assuming that selection acting through floral female success translates to selection on whole plants. We tested whether inflorescence height, flower display area, and corolla tube length are under selection at each site, and, in particular, whether any portion of the selection is mediated by pollinators. To quantify the phenotypic traits, we selected 480 plants at each site (except $n = 240$ at Lumle because of a low density of flowers) following the sequence of anthesis at each site, subject only to the requirement that plants had mature floral buds about to open and occurred as pairs within 50 cm of each other. Inflorescence height (height from the ground to the topmost part of a flower), floral diameter (distance between the labellum and staminodes; Fig. 2d) and breadth (width of the labellum at the widest point; Fig. 2d), and corolla tube length (distance from the top of the ovary to the entrance of the tube; Fig. 2f) were measured to the nearest millimetre. An index of floral display area was estimated by multiplying the floral diameter and breadth.

Half the selected plants were randomly assigned to a natural pollination treatment and half to a supplemental hand-pollination treatment, in such a way that each treatment was applied to one of the two plants occurring in pairs within 50 cm of each other. For natural pollination, plants were left untouched while for the supplementary treatment stigmas were covered with pollen collected from a donor plant at least 5 m away. Supplemental hand pollination removes any phenotypic selection imposed by pollinators by providing all plants with sufficient pollen for full fruit and seed set regardless of their phenotypic trait values. After *c.* 30 d, fruits were collected and the rate of fruit set and seed numbers per flower were evaluated. Flowers that did not set fruit were evaluated as zero fruit and seed.

To estimate phenotypic selection, trait values (inflorescence height, floral display area and corolla tube length) at each site were separately standardized to unit variance and centred on zero. Fitness proxies (fruit set and seed set) were also separately relativized for each site by dividing individual fitness values by mean fitness. Selection on a given trait was estimated through multiple regression models with relativized fitness as a response variable and standardized trait values as predictors (Lande & Arnold, 1983). Directional selection was estimated using the standardized partial regression coefficient (β) of a linear effect in the generalized linear model (GLM), while stabilizing or disruptive selection gradients (γ) were calculated by doubling the nonlinear (quadratic) partial regression coefficient obtained from the GLM (Stinchcombe *et al.*, 2008; Parachnowitsch & Kessler, 2010; Lavi & Sapir, 2015).

The selection gradient obtained from natural pollination represents the strength of selection through all factors, while the selection gradient in a supplementary pollination treatment excludes the selection component imposed by pollinators (Lavi & Sapir, 2015). The difference between selection gradients in the two treatments indicates the net selection imposed by pollinators (Sletvold & Agren, 2010; Sletvold *et al.*, 2010). We used ANCOVA to examine the difference in the selection gradients between the two treatments, using standardized trait values as continuous covariates, the fitness proxy as the response variable, and pollination treatments as the categorical factor. The covariate-by-factor interaction term in such an ANCOVA indicates differences in the selection gradients between two treatments and, if significant, provides evidence in favour of pollinator-mediated selection (Lavi & Sapir, 2015). Such a result demonstrates half of the reciprocal selection that potentially occurs in the *R. purpurea–P. longirostris* interaction.

Reciprocal selection

We also directly assessed the strength and direction of mutual selection between *R. purpurea* and *P. longirostris*. The time requirements for such detailed experiments limited the work to two of the five sites. Experiments were conducted at Daman and Mudhe (Table S1) from 2 to 25 August 2014 when plants were densely flowering. Forty mature flower buds at each site were covered with mesh bags. Following anthesis, each experimental flower was cleared of self-pollen by gentle brushing, following the method of Fan & Li (2012). The bract that covers the corolla tube was carefully removed and nectar level in each corolla tube was marked with a pen. The flowers were then exposed to pollinators. Flies visiting the experimental flowers were allowed free access to the nectar and were captured with a net as they departed. The visited flower was carefully removed, its corolla tube length was measured, and the level of remaining nectar in the corolla tube was marked. Nectar consumption by the fly was calculated from the change in the nectar level and expressed in terms of percentage consumption. The stigma was placed in 1.5 ml of 70% alcohol and brought to the lab to count the pollen

grains deposited on the stigma. The pollen grains were suspended in the solution by shaking and the number of grains in 20 µl of this suspension was counted under a microscope following the methods outlined in Dafni *et al.* (2005). Proboscis length of the trapped fly (linear distance from the base to the distal end of the prementum) was measured to the nearest millimetre following the method of Morita (2011). To prevent the same fly from foraging on other experimental flowers, captured flies were retained in a mesh bag. Upon completion of the experiment, all but voucher specimens of the collected flies were released.

Nectar consumption and pollen deposition were used as fitness proxies and were regressed against fly proboscis length and corolla tube length in GLMs. We used regression models including linear and quadratic terms to estimate linear and nonlinear selection gradients following Lande & Arnold (1983) and Stinchcombe *et al.* (2008). Before analysis, fitness values were relativized to the mean and traits values were standardized to unit variance and centred on zero (Lande & Arnold, 1983). The standardized linear regression coefficient of nectar consumption on proboscis length estimated the directional selection acting on *P. longirostris*, while the regression coefficient of stigmatic pollen load on corolla tube length estimated selection on *R. purpurea* (Alexandersson & Johnson, 2002; Pauw *et al.*, 2009). To estimate stabilizing or disruptive selection gradients, nonlinear regression coefficients (quadratic terms) were doubled (Stinchcombe *et al.*, 2008; Lavi & Sapir, 2015). In order to estimate how any selection on plant or pollinator revealed in these analyses depended on the mechanical fit between corolla tube and fly proboscis, we computed GLMs of the relationship between the percentage of nectar consumption or pollen deposition as dependent variables and the difference between corolla tube length and pollinator proboscis length as the independent variable.
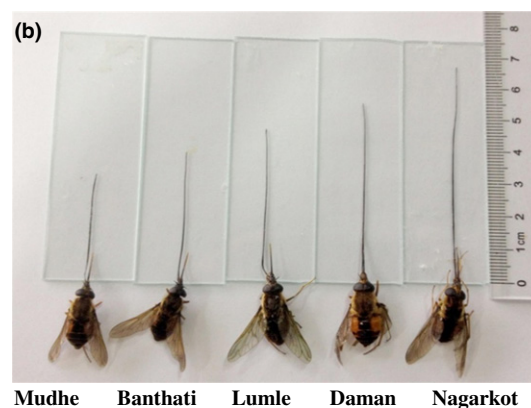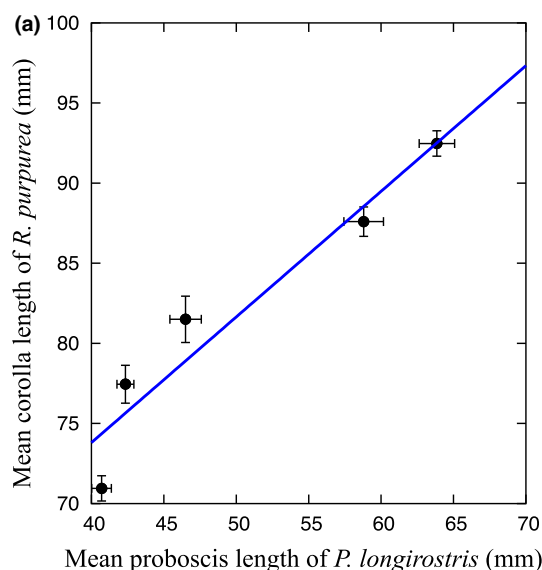
All statistical analyses were computed using R v.3.0.2 (R Development Core Team, 2013).

Results

Geographical covariation of interacting traits

Average corolla tube length of *R. purpurea* ranged from 70.9 ± 0.8 mm to 92.5 ± 0.8 mm (mean $\pm$ SE) among the five sites (Fig. 1). Differences among sites were significant (one-way ANOVA: $F = 63.662$; $P < 0.001$). Similarly, average proboscis length of *P. longirostris* varied from 40.70 ± 0.7 mm to 63.9 ± 1.3 mm (Fig. 1) and differed significantly among sites (one-way ANOVA: $F = 99.601$; $P < 0.001$). Most importantly, corolla length and proboscis length were significantly correlated across the five sites ($\beta = 0.960$; $t = 5.909$; $P < 0.05$; Fig. 3). Such a correlation is consistent with reciprocal selection occurring locally in individual populations.

Corolla tube length had no significant relationship with plant height or flower size ($P > 0.05$; Table 1). Similarly, fly proboscis length did not show a significant relationship with body length or width ($P > 0.05$; Table 2). Furthermore, there were no significant relationships of either corolla tube length or fly proboscis length with latitude, longitude or altitude (Mantel tests:

Fig. 3 (a) Covariation between corolla tube length of *Roscoea purpurea* and proboscis length of *Philoliche longirostris* across the five study populations. Error bars represent $\pm$ 1 SE. (b) Illustration of proboscis length variation of *P. longirostris* across the study sites (see Fig. 1 for site locations). The order of sites from left to right in (a) resembles the left to right order in (b) and corresponds to increasing mean proboscis length/corolla tube length in the sites.

Table 1 Test of allometric relationships of corolla tube length with plant height and flower size in *Roscoea purpurea* at different sites

Site	Relation with plant height			Relation with floral display area		
	β	t	P	β	t	P
Lumle	0.088	0.263	0.794	0.007	1.562	0.127
Nagarkot	0.167	0.848	0.402	0.003	0.153	0.880
Banthati	−0.316	−1.330	0.192	−0.003	−0.682	0.499
Daman	0.312	1.736	0.091	0.002	0.447	0.658
Mudhe	0.267	1.459	0.153	−0.001	−0.764	0.450

The table gives regression coefficients of linear models with plant height and floral display area as predictive variables for corolla tube length. Sample size: $n = 40$ for each site.

$P > 0.05$; Table S2). That is, the variation among sites in corolla tube length and proboscis length is not a consequence of underlying variation in plant size, flower size, or fly body size,

Table 2 Test of allometric relationships of proboscis length with body size in *Philoliche longirostris* across different sites

Site	Relation with body length			Relation with body width		
	β	T	P	β	t	P
Lumle	−0.8282	−0.678	0.507	−1.2515	−0.781	0.445
Nagarkot	0.107	0.102	0.920	1.846	1.093	0.290
Banthati	−0.1315	−0.287	0.777	0.329	0.489	0.631
Daman	1.269	0.978	0.342	0.849	0.389	0.702
Mudhe	−0.304	−0.301	0.767	0.369	0.338	0.739

Body length and thorax width of the fly are predictive variables and proboscis length is the response variable in linear models. Sample size: $n = 20$ at each site.

nor is it attributable to a common response of tube length and proboscis length to gene flow or environmental factors represented by geographical distance or altitudinal difference between sites.

Selection on floral traits

We found consistent, significantly positive relationships between plant fitness and corolla tube length at all sites (Table 3). By contrast, there was inconsistent evidence among naturally pollinated flowers of directional selection on inflorescence height and floral display, the advertising traits. Significant selection gradients for inflorescence height occurred at only two sites, while directional selection gradients for floral display area were negative at one site and nonsignificant at the remaining four sites (Table 3). Both fitness proxies, fruit set and seed number, yielded highly concordant results, and we present only seed number analyses in Table 3, while fruit set results are given in Table S3.

As expected, supplementary hand pollination of flowers erased the signature of pollinator-mediated selection that occurred in the naturally pollinated treatment (Tables 3, S3). Fitness had no relationship to the plant traits when pollen was supplied artificially, while fitness consistently increased with corolla tube length when flies delivered pollen (Fig. 4 for Daman and Mudhe; Fig. S1 for the remaining sites). Differences in the selection gradients between the two treatments were significant at every site, as indicated by the ANCOVA interaction term for linear effects (Table 3). Interactions between inflorescence height and treatment were significant at all but one site, while the interaction between floral display area and treatment was significant at only one site (Table 3).

Neither stabilizing selection (negative coefficient for γ) nor disruptive selection (positive coefficient for γ) seemed widespread or important for any of the three traits, inflorescence height, floral display, or corolla tube length (Tables 3, S3).

Reciprocal selection

The mean corolla tube length of *R. purpurea* flowers was longer than the mean proboscis length of flies at each of the five sites. On average, therefore, flies were forced to probe the flowers deeply to obtain nectar. At both Daman and Mudhe, the more the tube length exceeded the proboscis length in individual floral visits, the greater the stigmatic pollen deposition, on average (Fig. 5; Daman: β = 0.224; $t = 4.356$; $P < 0.001$; Mudhe: β = 0.109; $t = 2.345$; $P = 0.030$). By contrast, the percentage of nectar consumed by flies decreased as tubes became longer than proboscides (Fig. 5; Daman: β = −0.164; $t = −5.191$; $P < 0.001$; Mudhe: β = −0.096; $t = −3.712$; $P = 0.001$). Thus, a greater difference between corolla tube length and proboscis length was beneficial for plants while a smaller difference was beneficial for

Table 3 Selection gradients on floral traits of *Roscoea purpurea* via seed number per flower

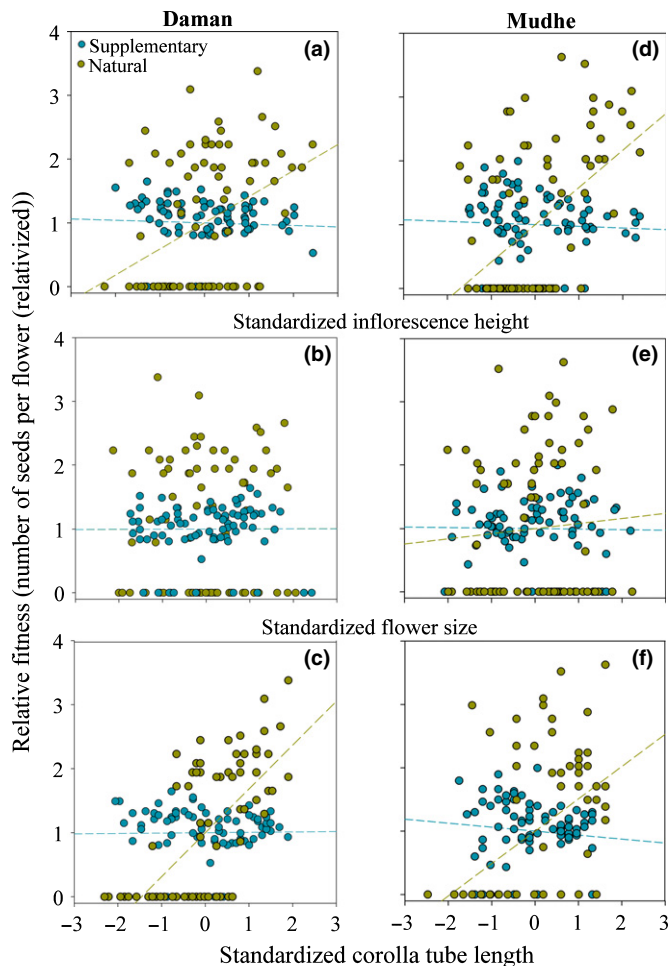| Sites	Trait	β, natural	β, supplementary	Interaction P-value	γ, natural	γ, supplementary	Interaction P-value
L	IH	0.164 ± 0.124	−0.066 ± 0.081	**<0.001**	−0.468 ± 0.910	−0.208 ± 0.516	0.833
	FS	**−0.278 ± 0.101**	0.087 ± 0.061	**0.004**	−0.584 ± 0.905	**1.475 ± 0.523**	**0.006**
	CTL	**0.579 ± 0.125**	0.039 ± 0.080	**<0.001**	−0.353 ± 0.915	0.539 ± 0.510	0.632
N	IH	−0.124 ± 0.113	−0.092 ± 0.089	0.824	−0.738 ± 0.730	−0.443 ± 0.485	0.389
	FS	0.066 ± 0.111	−0.027 ± 0.074	0.413	−0.259 ± 0.736	0.663 ± 0.462	0.719
	CTL	**0.490 ± 0.112**	0.090 ± 0.089	**0.001**	0.154 ± 0.844	**−0.928 ± 0.445**	0.482
B	IH	**0.297 ± 0.137**	0.024 ± 0.097	**0.044**	−0.214 ± 0.872	−0.260 ± 0.423	0.533
	FS	−0.016 ± 0.134	0.014 ± 0.063	0.396	−0.967 ± 0.839	0.393 ± 0.391	0.123
	CTL	**0.563 ± 0.139**	0.135 ± 0.100	**0.001**	0.0421 ± 0.890	−0.003 ± 0.413	0.693
D	IH	0.064 ± 0.114	−0.044 ± 0.063	**<0.001**	0.236 ± 0.802	0.359 ± 0.413	0.597
	FS	−0.114 ± 0.094	0.004 ± 0.048	0.995	0.114 ± 0.792	−0.693 ± 0.413	0.297
	CTL	**0.665 ± 0.110**	0.035 ± 0.063	**<0.001**	1.563 ± 0.808	0.765 ± 0.420	0.496
M	IH	**0.485 ± 0.115**	0.004 ± 0.064	**<0.001**	**2.323 ± 0.971**	0.175 ± 0.519	**<0.001**
	FS	0.007 ± 0.111	−0.012 ± 0.058	0.541	−0.603 ± 0.979	−0.414 ± 0.511	0.418
	CTL	**0.386 ± 0.114**	−0.066 ± 0.064	**<0.001**	0.350 ± 0.955	−1.016 ± 0.537	0.975

IH, inflorescence height; FS, flower size; CTL, corolla tube length. Linear selection gradients (β) represent linear regression coefficients obtained through the generalized linear model (GLM). Nonlinear selection gradients (γ) were obtained by doubling the quadratic regression coefficients. Positive and negative values of γ represent disruptive and stabilizing selection, respectively (Lavi & Sapir, 2015). Gradients are presented ± 1 SE. Values in boldface represent significant differences from zero at $P < 0.01$. Interaction P-values test the homogeneity of slopes between natural and supplemental pollination treatments in an ANCOVA model. Significant interactions imply a difference in selection gradients attributable to pollination treatments and thus significant pollinator-mediated selection. Sites: L, Lumle; N, Nagarkot; B, Banthati; D, Daman; M, Mudhe. Sample size: $n = 40$ at each site.

Fig. 4 Test for pollinator-mediated directional selection on floral traits of *Roscoea purpurea*. (a–c) Results at Daman (D); (d–f) results at Mudhe (M). Blue lines and circles, supplementary pollination; yellow lines and circles, natural pollination. See Table 3, sites D and M, for significance of slopes and of interactions testing heterogeneity of slopes.

pollinators. Moreover, pollen deposition increased with corolla tube length at Daman ($\beta = 0.545$; $t = 2.760$; $P < 0.05$) and at Mudhe ($\beta = 0.629$; $t = 3.430$; $P < 0.005$) (Fig. 6a), and nectar consumption increased with proboscis length at Daman ($\beta = 0.789$; $t = 5.443$; $P < 0.001$) and at Mudhe ($\beta = 0.661$; $t = 3.736$; $P < 0.001$) (Fig. 6b). Linear directional selection gradients were therefore significantly positive on both tube length and proboscis length at both sites, but there was no evidence of stabilizing or disruptive selection ($P > 0.05$; Table S4).

Discussion

Geographically variable interactions

Our results demonstrate localized geographical covariation between the corolla tube length of *R. purpurea* and the proboscis length of *P. longirostris*, similar to several other such instances of geographical co-variations (Steiner & Whitehead, 1990; Pauw *et al.*, 2009; Cosacov *et al.*, 2014; Newman *et al.*, 2014). Abiotic factors may play a role in the geographical

structure of covarying traits (Anderson & Johnson, 2008), but we did not find a significant relationship of traits with geographical distance or altitude (Table S2), surrogates for the abiotic environment and genetic kinship between populations (Anderson & Johnson, 2008; Newman *et al.*, 2014). The covariation of interacting traits is consistent with the geographical mosaic theory of coevolution, which argues that selection at the local level plays a significant role in determining the extent of adaptation (Thompson, 2005). The strength of selection varied among sites (Table 3). The geographical mosaic theory allows for the existence of localized hotspots with reciprocal selection and coldspots with one-way or absent selection. We could directly examine selection in both directions at only two sites, and in both instances selection was reciprocal (Fig. 5). Supplemental pollination experiments showed significant directional selection on corolla tube length at the three other sites (Table 3). In the absence of direct examination of selection on visiting flies, it remains possible that these site are coldspots with one-way selection. The absence of evidence does not exclude them as potential hotspots, however, and the geographical correlation of tube and proboscis lengths across five sites is consistent with reciprocal selection at all five sites. Across the current study sites, we did not find other pollinators that can substitute *P. longirostris* nor other plants that serve as alternative food sources for *P. longirostris*, circumstances that would allow coevolutionary coldspots to arise. Such coldspots could occur elsewhere in the ranges of these species, but ubiquitous hotspots are concordant with the finding of Benkman *et al.* (2003).

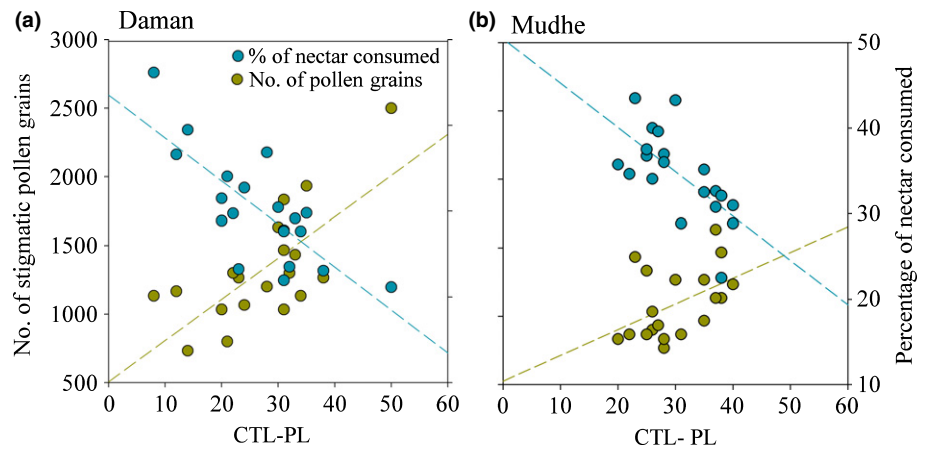### Weak or absent selection for advertising traits

We found some evidence of pollinator-mediated selection on inflorescence height in *R. purpurea*, but in only a minority of sites. The underlying mechanism for such selection could be greater pollinator attention to flowers that are part of larger inflorescences, but the inconsistency in our results implies that pollinator-mediated selection is not the most important factor affecting inflorescence height. Environmental factors such as resource availability, soil composition and moisture, and precipitation are likely to have a far more important effect on inflorescence display size. The abiotic environment of our sites, as summarized in latitude, longitude, and altitude, had no significant effect on inflorescence height, but other specific environmental factors probably play an important role.

Floral display area also seemed to be subject to little or no pollinator-mediated selection, despite the obvious role of flower size as an advertisement. It is possible that, in the densely flowering populations of *R. purpurea* that we studied, the signal of individual floral size is overwhelmed by inflorescence size or patchiness in the density of inflorescences.
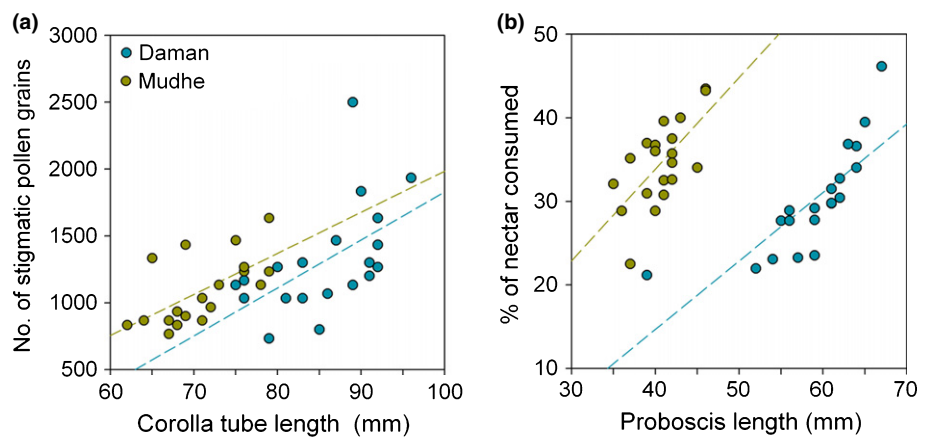
Evidence for reciprocal coevolution

The two putatively interacting traits covaried across the landscape in a manner consistent with the action of locally variable

Fig. 5 Variation in fitness benefit between plants and pollinators with variation in the relative lengths of interacting traits. CTL-PL refers to the difference between corolla tube length of *Roscoea purpurea* and proboscis length of *Philoliche longirostris*. With an increase in the CTL-PL value, the number of pollen grains deposited increases while the percentage of nectar consumed decreases. Yellow lines and circles, the number of pollen grains deposited; blue lines and circles, the percentage of nectar consumed during the same foraging bout of a fly to a flower of *R. purpurea*. (a) The interaction between *R. purpurea* and *P. longirostris* at Daman; (b) the interaction at Mudhe.

Fig. 6 Reciprocal selection between corolla tube length of *Roscoea purpurea* and proboscis length of *Philoliche longirostris*. Blue circles and lines, the relationship at Daman; yellow circles and lines, the relationship at Mudhe. (a) Proboscis length of the fly imposed directional selection on corolla tube length via the number of pollen grains deposited. (b) Corolla tube length of *R. purpurea* imposed directional selection on the proboscis length of *P. longirostris* through nectar consumption.

reciprocal selection (Fig. 3). Direct examination of selection was also consistent with geographically variable coevolution. As in several previous studies (Nilsson, 1988; Alexandersson & Johnson, 2002; Anderson & Johnson, 2009; Anderson *et al.*, 2009), our results with *R. purpurea* show strong signs of pollinator-mediated selection on corolla tube length at every site (Table 3). We found positive relationships of female fitness with corolla tube length at every site, but independence of tube length from allometric relationships with floral display area or inflorescence size (Table 1), and independence from relationships with abiotic factors summarized by latitude, longitude, and altitude (Table S2). These results suggest that the evolution of tube length is likely to depend on the selection pressure imposed by the pollinators. The paucity of evidence for stabilizing or disruptive selection on tube length (Table 3) suggests that plants always benefit from having corolla tubes long enough to force all or nearly all pollinators to probe flowers deeply, thereby pressing on the basal staminal appendages to effect pollen transfer.

We can further support the case for reciprocal selection from measurements made during individual floral visits. The more corolla tube length exceeded fly proboscis length, the greater the resulting plant fitness (judged by the proxy measure of pollen deposition) and the more fly visitors were disadvantaged (judged by their nectar consumption) (Fig. 5). The net effect is that both corolla tubes and fly proboscides appear to be under directional

selection for greater length (Fig. 6). The logistical demands of the work allowed us to examine reciprocal selection directly at only two sites, but at both sites the evidence strongly supported the existence of reciprocal selection.

Within the range we studied, *R. purpurea* served as a major, possibly the only, food source for *P. longirostris* and, in turn, the fly was the exclusive visitor to *R. purpurea* flowers (Sen, 1931; Paudel *et al.*, 2015). Thus, each actor in the relationship is potentially under selection pressure imposed by the other (Pauw *et al.*, 2009). Across the five study sites, corolla tube length of *R. purpurea* and proboscis length of *P. longirostris* varied concordantly (Fig. 3), consistent with the hypothesis of reciprocal coevolution. The outcome of this interaction appears to have been a mosaic of local coevolutionary races, leading to trait exaggeration of different degrees. That each site reached a different endpoint of the interaction could be a result of different strengths of interaction or selection among sites, random differences in trait values at the outset of the interaction that lead to different endpoints under the same dynamics, or unknown factors that impose selection on corolla tube length or fly proboscis length. Visitation rates of flies, and thus the strength of the plant–pollinator interaction, varied among sites (Paudel *et al.*, 2015). Selection strength certainly varied among sites (Table 3). The lack of association of trait values with geography and altitude (Table S2) implies that highly variable local factors could influence the

outcome of the coevolutionary dynamics, through either chance variation in average trait values among interacting populations or unknown sources of selection acting in addition to reciprocal selection.

The corolla tubes of *R. purpurea* are, on average, 30–40% longer than the proboscides of *P. longirostris* at any given site. Such mismatches in average trait values appear to be a common outcome of reciprocal plant–pollinator interactions, with the plant traits typically more exaggerated than the pollinator traits (Nilsson, 1988; Pauw *et al.*, 2009; Anderson *et al.*, 2010). The degree of mismatch in the *R. purpurea*–*P. longirostris* system is larger than the average mismatch among the 137 plant–insect interactions compiled by Anderson *et al.* (2010), but still well within the range observed in their sample. Within the *R. purpurea*–*P. longirostris* system, variation around the trait averages in each population creates a range of mechanical fits for individual interactions between flowers and fly visitors (Fig. 5). Such variation is compatible with the directional selection gradients we found. The strength of selection is unlikely to be symmetrical for plant and pollinator, however. Flies visiting a flower with a relatively long corolla tube will obtain less nectar than they would with a more favourable match, but a poorly rewarded visit is likely to be only one of many, and flies with shorter proboscides may depart flowers more quickly and thus visit more flowers per unit foraging time. The overall effect of mismatch on lifetime fly fitness may thus be small. Plants, by contrast, will always benefit from forcing their visitors to probe deeply for nectar, as the pollen transfer is not assured merely by the presence of a visiting fly on the labellum of *R. purpurea*. Only when a fly pushes its head into the corolla tube does it contact the lever-like staminal appendages that effect pollen transfer. A supplemental pollination experiment showed that seed output of *R. purpurea* is pollen-limited at every site (Paudel *et al.*, 2015; B.R.P. & Q-J.L., unpublished data), so that the fitness cost to the plant of a forgone mating opportunity may be greater than the cost to the fly of missing part of a meal. This asymmetry mirrors the 'life−lunch' asymmetry of predator−prey systems and may account for the greater elaboration of plant traits than insect traits in our system and in plant–insect interactions in general (Anderson *et al.*, 2010; Thompson, 2013).

Several alternative explanations for the elongation of interacting traits in our plant–pollinator mutualism seem less likely than a reciprocal coevolutionary race. Corolla tube elongation can be associated with a pollinator shift (Whittall & Hodges, 2007; Tripp & Manos, 2008), but this is unlikely in our case as we did not find any other pollinators of *R. purpurea* across the entire range of sites. Moreover, the pollinator shift hypothesis best applies either in the initial stage of tube length elongation or to elongation during speciation (Whittall & Hodges, 2007; Muchhala & Thomson, 2009). It has been proposed that long proboscides of floral visitors may evolve in order to evade predators at the floral surface (Wasserthal, 1997). Elongated proboscides allow a visitor to avoid its sit-and-wait predators by allowing nectar feeding from a distance while aerial predators are foiled by 'swing hovering' during nectar feeding. However, we did not observe any predators on the flowers of *R. purpurea* nor did we observe hovering feeding by *P. longirostris*: during every foraging bout, the fly lands on the flower.

Conclusions

Obligate specialized pollination in *R. purpurea* allows pollinator-mediated selection through female function to influence the evolution of corolla tube length. In turn, tube length appears to impose selection pressure on the pollinator's proboscis length, thus creating a coevolutionary interaction between *R. purpurea* and *P. longirostris*. Our results suggest that the reciprocal dynamics of this interaction occur locally in individual populations, leading to a mosaic of local adaptation.

Author contributions

B.R.P. and Q-J.L. designed experiment. B.R.P. conducted field/lab work. B.R.P., M.S., M.B., S.A., Y-S.S. and Q-J.L. analyzed the data, interpreted the results, and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 Test for pollinator-mediated directional selection on floral traits of *R. purpurea* at three study sites (Lumle, Banthati and Nagarkot).

Table S1 Geographical details of field sites and spatial covariation between corolla length of *R. purpurea* and proboscis length of *P. longirostris*

Table S2 Relationship between pair-wise geographical distance and altitude with corolla tube length of *Roscoea purpurea* and proboscis length of *Philoliche longirostris*; as revealed by Mantel Test

Table S3 Selection gradients for inflorescence height, floral display area and corolla tube length of *R. purpurea* via fruit set proportion per flower

Table S4 Nonlinear (stabilizing or disruptive) selection gradients for corolla tube length of *R. purpurea* and proboscis length of *P. longirostris*

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