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Reproductive isolation in alpine gingers: How do coexisting *Roscoea* (*R. purpurea* and *R. tumjensis*) conserve species integrity?

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Multiple barriers may contribute to reproductive isolation between closely related species. Understanding the relative strength of these barriers can illuminate the ecological factors that currently maintain species integrity and how these factors originally promoted speciation. Two Himalayan alpine gingers, *Roscoea purpurea* and *R. tumjensis*, occur sympatrically in central Nepal and have such similar morphology that it is not clear whether or how they maintain a distinct identity. Our quantitative measurements of the components of reproductive isolation show that they are, in fact, completely isolated by a combination of phenological displacement of flowering, earlier for *R. tumjensis* and later for *R. purpurea*, and complete fidelity of visitation by different pollinator species, bumblebees for *R. tumjensis* and a long-tongued fly for *R. purpurea*. Furthermore, the nectar of *R. tumjensis* flowers is available to the shorter tongued bumblebees while *R. purpurea* nectar is less accessible, requiring deep probing from long-tongued flies. Although flowering phenology is a strong current barrier that seemingly obviates any need for pollinator discrimination, this current pattern need not reflect selective forces occurring at the initial divergence of *R. tumjensis*. There has been considerable pollinator switching during the radiation of the Himalayan *Roscoea*, and the association of flowering time with type of pollinator in these sympatric species may have originated among the earliest or latest flowering individuals or populations of an ancestor to exploit either bumblebee activity early in the breeding season or long-tongued fly abundance later in the season. These two sympatric *Roscoea* species add to accumulating evidence of the primacy of prezygotic pollination traits in speciation among angiosperms even in the absence of postzygotic incompatibility.

KEY WORDS: **Bumblebee, floral color, long-tongued fly, pollination, speciation, sympatry.**

Coexisting congeneric plant species have often evolved traits to minimize interspecific reproductive interference and maintain their species integrity (Yang et al. 2007; Huang and Shi 2013). Sympatric congeners may possess several such reproductive barriers (Stone et al. 1996; Martin and Willis 2007; Liu and Huang 2013). Given the multiplicity of factors, knowing why and how those barriers arise is a major challenge in evolutionary biology (Dobzhansky 1937; Mayr 1942; Grant 1981; Coyne and Orr 2004).

Reproductive barriers can be broadly classified as prezygotic or postzygotic according to the timing of their occurrence (Grant 1981). Prezygotic barriers like phenological and floral isolation may reduce or prevent interspecific mating (Grant 1981, 1994; Hodges and Arnold 1994; Husband and Sabara 2003). Postzygotic

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barriers such as hybrid inviability or sterility impede gene flow even after heterospecific pollen transfer (Yang et al. 2007). The relative strengths of these barriers, how often single barriers are completely efficacious, the degree of asymmetry of isolation between species, and the distribution of total isolation strength among species are open questions concerning plant speciation (Lowry et al. 2008a). The sequential nature of isolating factors tends to give the earlier, prezygotic barriers a stronger effect than the later, postzygotic barriers on total reproductive isolation, even if they have equal individual strength (Coyne and Orr 1998; Ramsey et al. 2003; Nosil et al. 2005). Several recent studies have documented this disparity (Ramsey et al. 2003; Nosil et al. 2005; Kay 2006; Martin and Willis 2007; Yang et al. 2007; Lowry et al. 2008b), although Costa et al. (2007) found the opposite pattern in the *Chamaecrista desvauxii* complex.

Floral traits play a central role in prezygotic barriers (Grant 1994; Lowry et al. 2008a; Kay and Sargent 2009; Schiestl and Schlüter 2009). Features such as flower size, spur length, petal color, nectar level, and floral odor provide either mechanical isolation, in which floral morphologies are adapted to different pollinator taxa with different body shape or size, or ethological isolation, in which pollinators maintain behavioral fidelity to a species (Grant 1994; Schemske and Bradshaw 1999; Xu et al. 2011; Sun et al. 2015). Often the difference in floral traits between sympatric congeners is pronounced and obvious. For example, a color difference seems to be at the heart of prezygotic isolation between sympatric violet-flowered *Mimulus lewisii* (bee pollinated) and red-flowered *M. cardinalis* (hummingbird pollinated) (Ramsey et al. 2003). Purple-flowered, odorless *Petunia integrifolia* and white-flowered, scented *P. axillaris* differ in pollinator service from nocturnal hawkmoths, which visit only *P. axillaris* in their regions of sympatry (Dell'Olivo et al. 2011). In contrast, floral colors that differ dramatically between sympatric *Pedicularis rhinanthoides* and *P. longiflora* attract the same generalist *Bombus* pollinators to both species. In this case, where color fails to segregate pollinators, interspecific pollen transfer is sharply restricted by a difference in position of anthers and stigmas, so that pollen is dispensed and collected from different parts of the body of floral visitors (Yang et al. 2007). The variety of floral traits that can affect pollination, the need to measure them in meaningful ways (e.g., Kemp et al. 2015; Bukovac et al. 2017), and the idiosyncrasies of particular plant–pollinator interactions can make experimental study of prezygotic barriers difficult, particularly in the field. The difficulty of such assessments has limited the number and completeness of quantified measurements of species barriers in plants (Lowry et al. 2008a).

The alpine gingers *Roscoea purpurea* and *R. tumjensis* (Zingiberaceae) co-occur in central Nepal and display flowers of very similar appearance, to the point that the two species are sometimes mistakenly identified in published floras (Cowley and Wilford 1998) and many herbaria records. The genus *Roscoea* represents a recent shift to cooler upland habitats within the family Zingiberaceae, an otherwise largely lowland, tropical family (Kress and Specht 2006; Zhao et al. 2016). The genus occupies two distinct regions comprising two disjunct clades, the Himalayan clades occurring in the Himalayan slopes in the west and the Indo-Chinese clade, occurring in the highlands of northern Indochina and Yunnan Province in the east, a disjunction that arose from rapid eastward tectonic extrusion of Indochina at the Oligocene/Miocene boundary (Zhao et al. 2016). Molecular evidence suggests that *R. purpurea* is sister to a central Nepalese lineage that itself fragmented into three restricted endemic species, *R. tumjensis*, *R. capitata*, and *R. ganeshensis* (Ngamriabsakul et al. 2000). The order of speciation and nature of diversification among these three lineages is not fully resolved, but the close phylogenetic relationship between *R. purpurea* and *R. tumjensis* is not in doubt. Nonetheless, our field observations have failed to find any obvious natural hybrids between *R. purpurea* and *R. tumjensis* even though sympatric populations exist. How, then, is the species integrity of these sympatric and closely related gingers maintained?

In this study, we measured the strength of multiple isolation factors separating *R. purpurea* and *R. tumjensis*, and quantified their relative contribution in chronological order to total reproductive isolation following Sobel and Chen (2014). We identified the most important barriers between the two species and address here the traits responsible for isolation. Finally, because the estimation of relative contributions of different reproductive barriers can illuminate the situation under which speciation occurred (Ramsey et al. 2003; Coyne and Orr 2004; Kay 2006), we consider whether reproductive isolation between the two *Roscoea* species is linked to their speciation process.

Materials and Methods

STUDY SPECIES

Roscoea purpurea and *R. tumjensis* are small perennial herbs distributed in alpine slopes of the Himalayas. *Roscoea purpurea* has the wider range distribution, from Himachal Pradesh (India) in the west through Nepal to Bhutan and Assam (India) in the east at elevations of 1520–3100 m a.s.l., while *R. tumjensis* is more narrowly distributed and endemic to central Nepal from 2040–3050 m (Cowley 2007; personal observation) (Fig. 1). *Roscoea purpurea* tends to occur under canopies of *Pinus* and *Rhododendron* forest while *R. tumjensis* prefers open meadows, although both sympatric and allopatric populations occur in the area of overlap. Flowers of *R. purpurea* are light purple or white with purple markings to human vision, and appear only when the plant is fully matured. Flowers of *R. tumjensis* are light purple to human vision, and are produced before the leaves are fully emerged (Cowley 2007;

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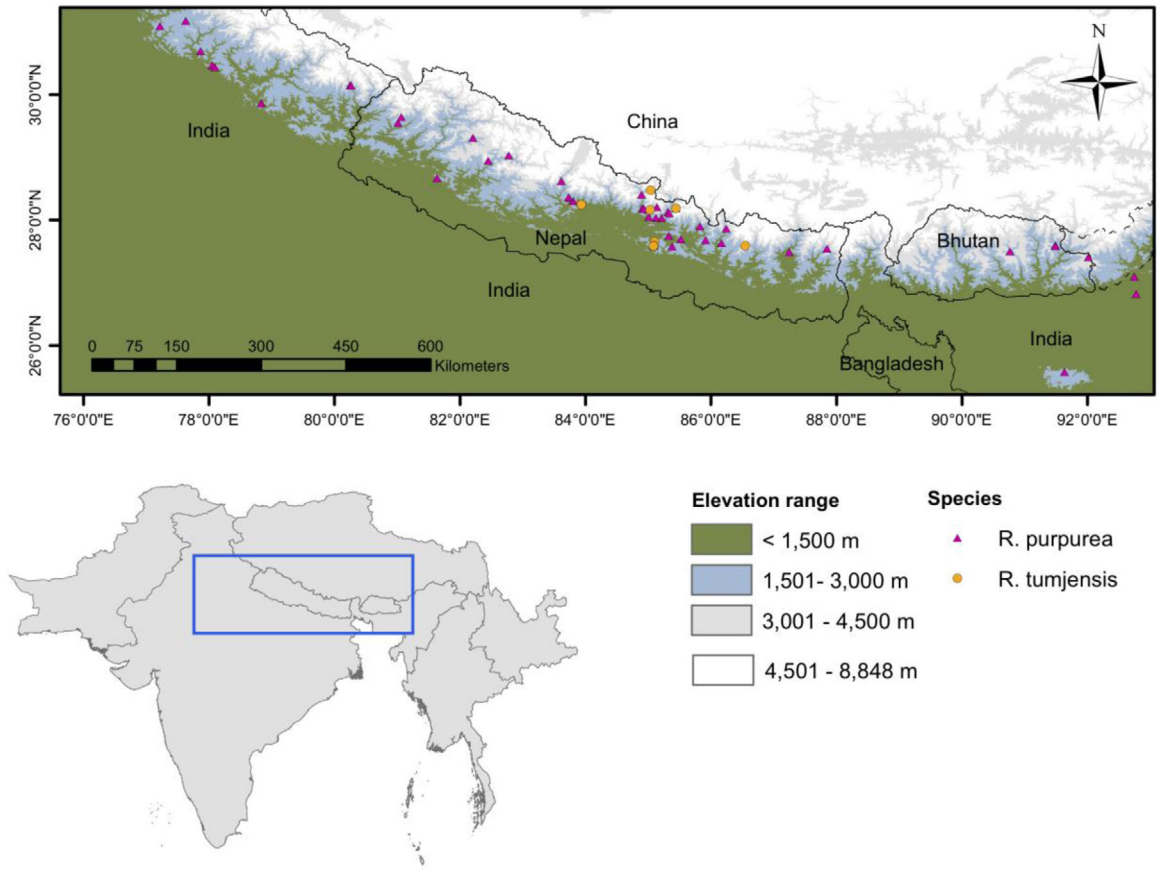

Figure 1. The entire distribution range of *R. purpurea* (magenta triangles, $n = 48$) and *R. tumjensis* (orange circles, $n = 8$). The distribution range of the two species was determined from literature (Cowley 1982, 2007; Cowley and Wilford 1998; Zhao et al. 2016), from records in the Global Biodiversity Information Facility (gbif.org), and by our extensive field surveys from 2012–2017.

Figure 2. A flowering individual of *Roscoea purpurea* (A), and its obligate pollinator, a long-tongued tabanid fly (*Philoliche longirostris*) (B). A flowering individual of *Roscoea tumjensis* (C), and its pollinators, the bumblebees *Bombus flavescens* (D) and *B. haemorrhoidalis* (E).

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BRP, personal observation) (Fig. 2). In both species, anthers are covered by staminodes and are located in the corolla throat just in front of the labellum. Removal and deposition of pollen occurs when a foraging visitor, in an attempt to draw nectar, pushes the lever-like mechanism of anther appendages, bending the stamen forward and releasing pollen grains on the back of the visitor. We know *R. purpurea* is pollinated by a long-tongued tabanid fly, *Philoliche longirostris* (Paudel et al. 2015, 2016), and the current study revealed *R. tumjensis* is pollinated by bumblebees.

STUDY SITES

Our field measurements were made in three sympatric populations in the Makawanpur district of central Nepal: Tistung (27° 39′ 36″ N, 85° 5′ 58″ E, 2014 m asl), Daman (27° 36′ 36″ N, 85° 5′ 37″ E, 2319 m asl), and Simbhangjyang (27° 35′ 27″ N, 85° 5′ 4″ E, 2475 m) (Fig. 1). Vegetation at all three sites consisted of mixed forest of *Pinus*, *Rhododendron* and *Quercus*.

GEOGRAPHIC ISOLATION

We estimated the geographic isolation between *R. purpurea* and *R. tumjensis* by comparing the overlap in their ranges. Locations of populations of the two species were determined from literature (Cowley 1982, 2007; Cowley and Wilford 1998; Zhao et al. 2016), from records in the Global Biodiversity Information Facility (gbif.org), and by an extensive field survey we conducted across the Nepalese Himalayas in the months of May–August from 2012 to 2016, yielding a total of 48 populations of *R. purpurea* and eight populations of *R. tumjensis*. *Roscoea tumjensis* is known to be endemic to Nepal with a very narrow distribution confined to central Nepal (Cowley 1982, 2007). Our extensive field surveys across the Nepal Himalayas have never uncovered populations outside this range. The total of eight populations of *R. tumjensis* in our sample provides a correct indication of geographic distribution. We calculated range areas from minimum convex polygons surrounding the populations, except that for *R. purpurea* we allowed concavity on the northern boundary to account for the geography of the Himalayan peaks that define the altitudinal limit of the species. Reproductive isolation due to geographic separation was calculated from equation 4C of Sobel and Chen (2014), $\mathrm{RI}_{\mathrm{geogr}} = 1 - S/(S + U)$, in which S is the fraction of space that is shared with the other species and U is the unshared fraction.

MICROHABITAT ISOLATION

We examined the fine-scale microhabitat isolation between the species by quantifying the degree of co-occurrence within small quadrats. In each of three years (2015, 2016, and 2017) at each of the three field sites with sympatric populations, we randomly placed 100 quadrats of 1 m × 1 m within a predefined one-hectare plot. We counted the number of quadrats containing only *R. purpurea*, only *R. tumjensis*, or both species. For each year we pooled data from the three sites and determined the proportion of quadrats that were shared and unshared for each species. From these proportions we calculated microhabitat isolation in each of the three years using equation 4C of Sobel and Chen (2014) (i.e., as for geographic range isolation but using the proportion of shared and unshared quadrat occurrences).

TEMPORAL ISOLATION

Isolation due to floral phenology was determined from samples of flower abundance of each species in five plots distributed among the three sympatric populations. Plots were sampled at five-day intervals from May to September 2013, 2014, and 2016. At each plot we randomly set out 20 quadrats of 1 m × 1 m and counted the number of open flowers of each species. We summed the flower counts across the five plots to obtain a flowering profile for each species in each year. We then calculated temporal isolation for each year from equation 4S2 of Sobel and Chen (2014), which accounts for the effect of floral abundance of each species on the probabilities of conspecific and heterospecific pollen transfer at each sample date.

POLLINATOR-MEDIATED FLORAL ISOLATION

We observed floral visitors to *R. purpurea* and *R. tumjensis* in natural and manipulated plots in field populations over several years. We had previously determined that neither species received any nocturnal floral visitors during 51 hours of night time monitoring while floral visitors were most active from 09:00 to 16:00 (Paudel et al. 2015). Further, flowers left exposed overnight from 19:00 to 06:00 the next morning but otherwise covered in mesh pollinator exclusion bags never set fruit (Paudel et al. 2015, and unpubl. data). We therefore concentrated on diurnal floral visitation.

In 2013 and 2014, we established four natural plots of 20 m × 20 m at each of which we observed pollinator arrivals and flower visitation for 12 h over 3 days during the peak blooming period of each species. Additionally, at one site we established two plots (10 m × 25 m) separated by more than 500 m and observed them at a time when both species were flowering (five individuals of *R. tumjensis* and 11 of *R. purpurea* in plot 1; eight individuals of *R. tumjensis* and six of *R. purpurea* in plot 2). Floral visitors were observed from 09:00 to 16:00 at both plots for five continuous days.

In 2016 and 2017 we created experimental mixed-species plots during the period of overlap in natural flowering between the two species. The plots were 3 m × 3 m and contained alternately placed flowering individuals of the two species at 1 m intervals, for a total of 16 plants per plot. Floral visitors in these arrays were observed from 10:00 to 16:00 over three days. We calculated pollinator-mediated isolation using equation 4A of Sobel and Chen (2014).

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POST-POLLINATION ISOLATION

Pollen-stigma interactions, pollen tube-style interactions, and maternal choice or competitive interactions among ovules can lead to postpollination incompatibility between species that is independent of embryo viability. We conducted reciprocal pollen transfer experiments to measure isolation arising at this stage. At each of the three sympatric populations, we covered a single bud per inflorescence from 40 individuals of each species with fine mesh bags to exclude natural pollinators. When those flowers opened, 20 flowers from each species received heterospecific pollen through hand pollination, and the remaining 20 flowers received intraspecific hand outcross-pollinations. The mesh bags were then replaced to exclude additional pollen deposition until the flowers wilted. After about 30 days, fruits from the experimental flowers were collected, opened, and numbers of filled seeds per fruit were counted. Flowers that did not set fruit were counted as having produced zero seeds; thus, calculation of mean seed set took account of fruit abortion. We calculated the postpollination isolation index based on seed formation following equation 4A of Sobel and Chen (2014), $RI_{postpollination} = 1 - 2[H/(H + C)]$, in which H and C are the mean seed number per fruit from heterospecific and conspecific pollinations.

POST-ZYGOTIC VIABILITY ISOLATION

Postzygotic isolation due to embryonic failure was calculated from the proportion of viable seeds produced by the interspecific and intraspecific crosses described under *Postpollination isolation*. We dissected the seeds of each treatment with a scalpel blade and observed the presence or absence of an embryo in each seed. We considered a seed as viable if it contained an embryo (Sun et al. 2015). The postzygotic isolation index was calculated from equation 4A of Sobel and Chen (2014) as for postpollination viability.

TOTAL REPRODUCTIVE ISOLATION

The combined effect of multiple barriers to interspecific reproduction must take account of the sequence in which barriers act, because mating impediments that act early in a breeding season limit the potential scope of action of subsequent barriers. We calculated the total reproductive isolation between the two species following equation 4E of Sobel and Chen (2014), which takes account of the separate ways in which spatial and temporal barriers, other prezygotic barriers, and postzygotic barriers contribute to total isolation. The relative contribution of the ith individual barrier, given its position in a sequence of n barriers, was calculated as $RC_i = (RI_i - RI_{i-1})/RI_{total}$, in which RI_i is the cumulative effect of the first i barriers to act and $RI_{total} = RI_n$.

FLORAL TRAITS

To explore the mechanisms underlying isolation due to pollinator fidelity, we quantified traits relating to detection (floral color and display) and access to rewards (corolla tube length and nectar availability).

To assess display size and reward traits, we randomly selected 20 plants of each species at all the three sympatric populations. We counted the number of flowers on each plant. On one flower from each plant we measured width of the labellum at the widest point, floral diameter (distance between labellum and top of corolla), corolla tube length (distance from the tube entrance to the top of the ovary), and nectar accessibility (distance from the tube entrance to the highest point of nectar accumulation in the tube), following the method of Paudel et al. (2015, 2016) (Fig. 3). The product of labellum width and floral diameter was used as a measure of individual flower area (Paudel et al. 2016). None of these traits within a species differed significantly among the three populations (one-way ANOVA, $P > 0.05$). The data from three sites were therefore pooled and the differences in floral traits between the two species were assessed using independent sample t-tests.

To assess floral color, we first measured the reflectance spectra of three flowers of each species collected from different locations in the study area. We recorded reflectance over wavelengths 300–650 nm using an Ocean Optics spectrophotometer (Dunedin, FL, USA) with a PX-2 pulsed xenon light source, calibrated with a UV-reflecting white standard (details in Dyer et al. 2012; Shrestha et al. 2013, 2016).

Results

GEOGRAPHIC ISOLATION

The entire range of *R. tumjensis* was enclosed within the *R. purpurea* polygon, while *R. purpurea* shared only 5.3% of its range with *R. tumjensis*. Geographic isolation for *R. tumjensis* was therefore $RI_{geogr,\ Rt} = 0$, that is, no isolation from *R. purpurea* at all, while geographic isolation for *R. purpurea* was $RI_{geogr,\ Rp} = 0.947$.

MICROHABITAT ISOLATION

At sites where the two species of *Roscoea* occurred, they co-occurred in approximately one-half to two-thirds of randomly placed 1 m × 1 m quadrats, depending on year (Table 1). At this spatial scale, then, microhabitat separation provided only modest reproductive isolation (Table 1). Averaged across the three years, the values were $RI_{microhabitat,\ Rp} = 0.254$ and $RI_{microhabitat,\ Rt} = 0.198$.

TEMPORAL ISOLATION

We found strong temporal separation in flowering periods of the two species. *Roscoea tumjensis* started to flower from the middle

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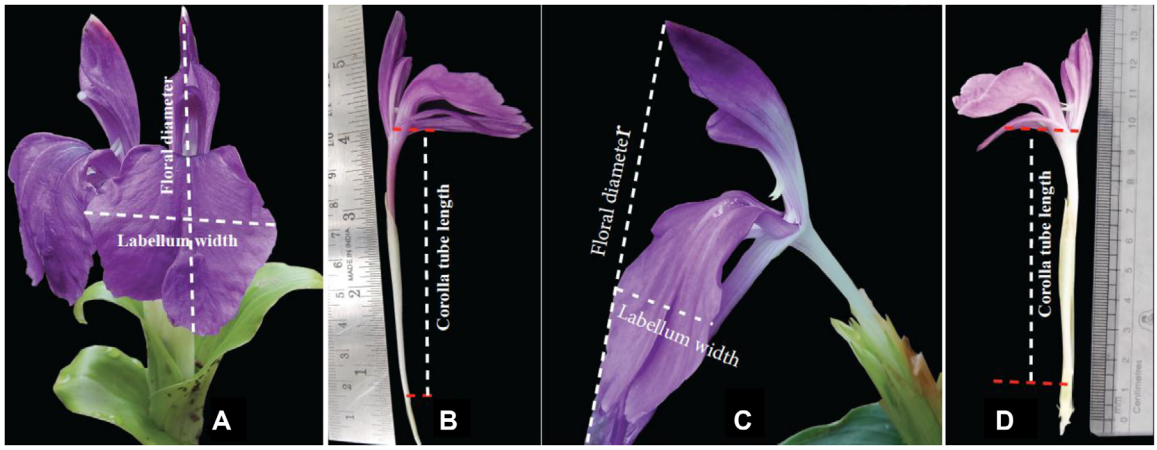

Figure 3. Floral traits measurements for the two *Roscoea* species: (A–B): *R. tumjensis* and (C–D): *R. purpurea.*

Table 1. Microhabitat (1 m × 1 m quadrat) occurrence and co-occurrence of the two *Roscoea* species at sites of sympatry between the species.

Year	No. quadrats with *Rp* only	No. quadrats with *Rt* only	No. quadrats with both species	$RI_{microhabitat,\ Rp}$	$RI_{microhabitat,\ Rt}$
2015	84	76	140	0.375	0.352
2016	56	23	221	0.202	0.094
2017	49	37	214	0.186	0.147

Rp = R. purpurea; Rt = R. tumjensis.

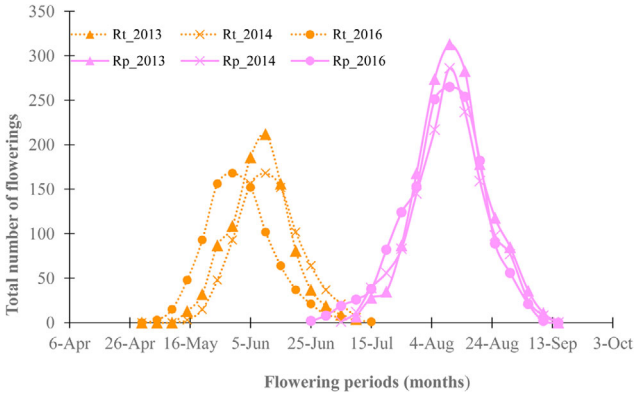

Figure 4. Flowering phenology of two *Roscoea* species for three years: *R. tumjensis* (orange color dotted line) and *R. purpurea* (magenta color solid line).

of May and persisted up to the middle of July, with a peak in early- to mid-June. *Roscoea purpurea* flowered from late June to early September, with a peak in mid-August. In each year, the blooming period of the two species overlapped for about 20 days when neither species had abundant flowers (Fig. 4). Temporal isolation between the species due to flowering phenology was therefore strong and similar in each direction, and consistent across years (Table 2). Averaged across the three years, the values were $RI_{temporal,\ Rp} = 0.983$ and $RI_{temporal,\ Rt} = 0.990$.

Table 2. Temporal isolation between the two *Roscoea* species due to flowering phenology.

Year	$RI_{temporal,\ Rp}$	$RI_{temporal,\ Rt}$
2013	0.995	0.997
2014	0.988	0.993
2016	0.968	0.981

The calculation of reproductive isolation takes account of floral abundance of each species, and thus opportunities for heterospecific pollen transfer, on days of overlapping flowering (Sobel and Chen 2014, equation 4SA).

POLLINATOR-MEDIATED FLORAL ISOLATION

Roscoea tumjensis flowers were visited by three types of diurnal foragers (Fig. 2), two species of bumblebees (*Bombus flavescens* and *B. haemorrhoidalis*) and a moth (*Macroglossum nycteris*). In a separate experiment, we determined that the moth was a nectar robber that did not affect pollen transfer (unpublished data). A long-tongued fly (*Philoliche longirostris*) was the only observed pollinator of *R. purpurea* across all sites (Fig. 2), a finding in accord with previous results (Paudel et al. 2015, 2016).

In the four natural plots observed in 2013 and 2014 during peak flowering of each species, 465 *B. flavescens* bees visited 2218 flowers and 510 *B. haemorrhoidalis* bees visited 2418 flowers of *R. tumjensis* over the cumulative 48 hours of observation

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time. During the later flowering peak of *R. purpurea*, we observed the arrival of 441 *P. longirostris* flies that visited 1308 flowers over 48 hours of observation. At the two natural plots observed during the overlap in flowering of the two species we failed to find any flower visitors, a result that may have been due to the low density of plants in flower at this time. In the experimental mixed-species plots, we did not see any pollinator visits in 2016, while in 2017 we observed 7 *Bombus* visitors that probed 23 flowers of *R. tumjensis* only, never of *R. purpurea*. This arrival and visitation rate of *Bombus* was comparable to that observed at peak flowering of *R. tumjensis* in the natural plots in 2013 and 2014, given the smaller area of the plots (9 m^2 compared to 400 m^2) and the shorter total observation time (18 h compared to 48 h). During all monitoring of plots as well as our broad observation throughout the three sympatric populations over several years of field work, we never observed any "mismatched" floral visits, that is, bee visits to *R. purpurea* or fly visits to *R. tumjensis*. Hence, we concluded that reproductive isolation between *R. tumjensis* and *R. purpurea* due to pollinator behavior was complete. The calculated value of $RI_{pollinator}$ was therefore unity for both species (Table 3).

POST-POLLINATION ISOLATION

Roscoea purpurea plants hand-pollinated with conspecific pollen had a fruit set rate of 0.82 and produced a mean of 31.5 seed per flower, and when hand-pollinated with *R. tumjensis* pollen had a fruit set rate of 0.78 and mean of 27.6 seeds per flower. For *R. tumjensis*, the equivalent results were a rate of fruit set of 0.85 and a mean of 34.1 seeds per flower under conspecific pollination, and 0.82 fruit set and mean seed number of 32.5 for heterospecific pollination. The similar values imply weak postpollination incompatibility between the species. Based on seed output, the strength of reproductive barriers was found as: $RI_{postpollination,\ Rp} = 0.066$ and $RI_{temporal,\ Rt} = 0.018$.

POST-ZYGOTIC VIABILITY ISOLATION

Conspecific hand pollination of 51 flowers of *R. purpurea* produced 1204 seeds of which 667 were viable (contained embryo), while hand pollination of 47 flowers with *R. tumjensis* pollen produced 1216 seeds, of which 635 were viable. Viability rates were thus very similar for the conspecific and heterospecific pollination treatments. Similarly, for *R. tumjensis*, 441 viable seeds from among 1170 total seeds were obtained from 45 fruits that had received conspecific pollen, and 529 viable seeds among 1211 total seeds from 49 fruits that had received heterospecific pollen. Again, the proportions of viable seeds were very similar for inter- and intraspecies crosses, pointing to weak incompatibility on the part of both species. The postzygotic reproductive isolation indices were $RI_{postzygotic,\ Rp} = 0.030$ for *R. purpurea* and $RI_{postzygotic,\ Rt} = -0.073$ for *R. tumjensis*. The latter value indicates a slight advantage for heterospecific embryos, although the proportion of viable seeds did not differ significantly between *R. tumjensis* fruits from inter- and intraspecific crosses (independent sample *t*-test, $t = -1.077$, d.f. $= 121$, $P = 0.28$).

TOTAL REPRODUCTIVE ISOLATION

The individual effects of the four pre-pollination barriers and two postpollination barriers are summarized for each species in Table 3. The cumulative effect of these barriers nearly reached unity at the prezygotic stage due to spatial and temporal separation of the two ginger species (Table 3). Although pollinator fidelity appeared to be absolute (*R. purpurea* attracts long-tongued flies and *R. tumjensis* attracts bumblebees), pollinator behavior made a small relative contribution to isolation of the two species, given the prior effects of isolation by geographic range, microhabitat preference, and flowering time. Given essentially complete reproductive isolation between the species up to the point of pollen deposition, postpollination and postzygotic barriers, which were

Table 3. Reproductive isolation (RI) and relative contribution (RC) of different reproductive barriers that contribute to the total isolation between *R. purpurea* and *R. tumjensis*.

	R. purpurea			*R. tumjensis*	
Barrier	RI	RC (complete)	RC (sympatry)	RI	RC
Geographic	0.947	0.947	—	0	0
Microhabitat	0.254	0.014	0.254	0.198	0.198
Temporal	0.983	0.039	0.733	0.990	0.794
Pollinator	1	0.001	0.013	1	0.008
Postpollination	0.066	0	0	0.018	0
Postzygotic	0.030	0	0	–0.073	0
Total	1	1	1	1	1

The mean value of RI is given here for Microhabitat, Temporal, and Pollinator isolation, which were assessed over multiple years. For *R. purpurea*, the RC of each barrier is given for the complete sequence of barriers and for a sequence excluding geographic isolation (i.e., barriers that act only in the zone of sympatry with *R. tumujensis*).

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Table 4. Floral display and nectar accessibility traits.

Trait	*R. purpurea*	*R. tumjensis*	*t*	*P*
No. flowers per plant	10.40 ± 0.43	4.74 ± 0.15	12.31	< 0.0001
Floral area (mm^2)	1557.5 ± 24.0	1014.2 ± 14.2	19.46	< 0.0001
Corolla tube length (mm)	89.3 ± 0.60	76.9 ± 0.78	12.67	< 0.0001
Distance to nectar (mm)	34.6 ± 0.54	18.2 ± 0.22	27.90	< 0.0001

Values are mean ± 1 S.E. See Methods for details of trait measurements. The differences in floral traits between the two species were assessed using independent sample *t*-tests.

weak or nonexistent in any case, made no contribution to species isolation (Table 3).

FLORAL TRAITS

Several display characteristics differed between the two species. *Roscoea purpurea* had inflorescences with about twice as many flowers and its individual flowers were about 50% larger than the inflorescences and flowers of *R. tumjensis* (Table 4). Although the display size of *R. purpurea* was therefore larger, its nectar reward was less accessible. Corolla tube length tended to be somewhat longer in *R. purpurea* than in *R. tumjensis*, but, more importantly, the mean distance from the tube opening to the nectar level was about 50% greater in *R. purpurea* than in *R. tumjensis* (Table 4).

Floral spectral reflectances (Fig. 5) suggest that a degree of divergence in chromatic cues may have emerged between the species, although there is also some overlap in spectral profiles. These results are preliminary and much more intensive sampling is required to characterize the variability and geographic pattern of floral color in these populations. However, using models of hymenopteran and dipteran color vision to interpret these spectra (see Shrestha et al. 2014, 2016 for examples of implementation), it suggests that bumblebee and fly pollinators could, depending on background and lighting conditions, distinguish the two species on floral color alone (unpublished results).

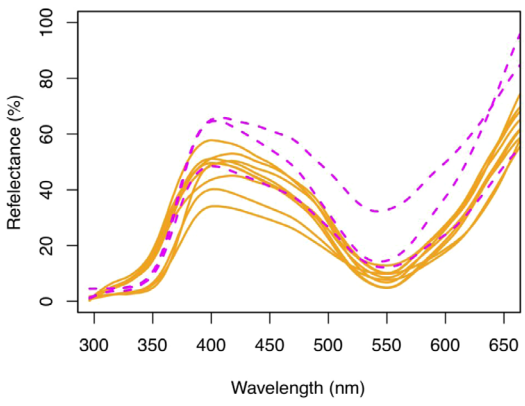

Figure 5. Spectral reflectance profiles of flowers of *Roscoea purpurea* (interrupted magenta curve) and *R. tumjensis* (solid orange curve).

Discussion

STRUCTURE OF EXTANT BARRIERS

We have shown that prezygotic barriers play a strong role in the total reproductive isolation between *R. purpurea* and *R. tumjensis*, while postzygotic barriers are nearly absent. Similar asymmetry is common in closely related sympatric species (Ramsey et al. 2003; Nosil et al. 2005; Kay 2006; Martin and Willis 2007; Lowry et al. 2008b; Christie and Strauss 2018). Among the prezygotic barriers, flowering schedules and strict pollinator specificity completely divide the two *Roscoea* species, even in the zone of sympatry between them (Table 3). Hence, interspecific pollen transfer between these two gingers is implausible. Christie and Strauss (2018) found substantially weaker, albeit still important, temporal RI among California Jewel flowers (mean $RI_{temporal}$ = 0.22), which appear to have much more generalized pollinator interactions. The strong association of phenology and pollinator identity in our sympatric *Roscoea* species may provide a clue to how their isolation was generated.

As in the case of sympatric *Pedicularis* species (Adams 1983; Yang et al. 2007), there is a substantial difference in peak blooming period between the two *Roscoea* species (Fig. 4). Their peak blooming times are coincident with the abundance of different pollinators. Our field observation indicated that the bee pollinators of *R. tumjensis* were active from May to mid-August while the tabanid fly pollinator of *R. purpurea* was most abundant throughout August, confirming previous reports (Sen 1931). Indeed, in the brief period of flowering overlap as *R. tumjensis* blooming wanes and that of *R. purpurea* begins, the winged stage of the fly pollinator of *R. purpurea* has not yet emerged, and these early flowering individuals often remain unpollinated.

Even when pollinators can make heterospecific floral visits, actual floral visitation patterns may segregate pollinators (ethological isolation), or floral morphology may impede heterospecific pollen transfer (mechanical isolation) (Grant 1994). As there were no heterospecific floral visits between the study systems, we were unable to measure potential mechanical isolation. Nonetheless, our results indicate that flowers of *R. purpurea* are adapted to a long-tongued fly and are avoided by other nonspecific pollinators, while flowers of *R. tumjensis* attract generalized pollinators

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but show specialization toward bumblebees for pollination success (Paudel et al., unpubl. data). Corolla tube length and nectar level would play a major role in preventing pollinator sharing between two species. *Roscoea purpurea* have longer corolla tubes and less accessible nectar than *R. tumjensis* (Table 4). Bumblebees would have difficulty extracting a reward from *R. purpurea*, and might even find it completely unrewarding. In contrast, the long-tongued tabanid fly *Philoliche longirostris* could reach *R. purpurea* nectar. Indeed, in previous work we have shown that reciprocal selection between *R. purpurea* and this pollinator has created a geographic mosaic of positively correlated corolla tube length and fly tongue length across Nepal (Paudel et al. 2016). The effect of reward accessibility is potentially asymmetric, as the long-tongued pollinator of *R. purpurea* would seem capable of reaching nectar in the shorter corolla tubes of *R. tumjensis*. Further experimentation could test this supposition and explore how reward accessibility affects ethological isolation. While other cases of complete or nearly complete ethological isolation are known (Ramsey et al. 2003), this may not be the rule among congeners (Coyne and Orr 2004).

Flowers of the two species, while similar enough to occasionally confuse botanists (Cowley and Wilford 1998), show some degree of difference in reflectance spectra. The floral color difference between *R. purpurea* and *R. tumjensis* is based largely on the intensity of reflection around 550 nm (Fig. 5). Similar pollinator discrimination on the basis of cryptic (to humans) color signals are known in *Mimulus guttatus* (Peterson et al. 2015).

ORIGIN OF REPRODUCTIVE BARRIERS AT INCIPIENT SPECIATION

The current importance of reproductive barriers need not reflect the historical order in which they arose nor the importance of their role in promoting speciation (Coyne and Orr 2004). Despite the apparently strong effect of geographic separation, it probably did not play a key role in speciation. While the wide range of *R. purpurea* across the Himalayas (a feature it shares with one other *Roscoea*, *R. alpina*) removes most of its populations from sympatry with *R. tumjensis*, *R. tumjensis* itself has probably always occurred within the range of *R. purpurea* (Ngamriabsakul et al. 2000; Cowley 2007), as reflected in its current geographic isolation index of 0 (Table 3). Such asymmetric range overlap may be common among recently derived species (Christie and Strauss 2018).

In the near absence of postpollination and postzygotic barriers, it seems likely that flowering time and pollinator attraction evolved together at the origin of divergence between *R. purpurea* and *R. tumjensis* (or the *R. tumjensis*/*R. capitata*/*R. ganeshensis* clade). Populations or individuals of the common ancestor flowering earlier or later than the activity of their typical pollinator could have been pollen limited (e.g., Forrest and Thomson 2010). Given the elevational variation and strong environmental gradients in Nepal and their effect on flowering times (Primack 2000), mismatches between pollinator abundance and blooming schedules in some populations are entirely plausible. If a new floral visitor were present at the margin of the ancestral flowering season, attraction of the alternative visitor might relieve pollen limitation and set up selection for traits that strengthen the new association. Selection would then favor a temporal shift in blooming to coincide with peak activity of the new pollinator, as well as signals and rewards that cater to its perception and foraging abilities. Shifts in flowering time of even a week can have strong isolating effects in some instances (e.g., Husband and Schemske 2000). Important shifts in floral color may be under simple genetic control (Glover and Martin 1998; Dyer et al. 2007) and play an essential part in pollinator shifts (Bradshaw and Schemske 2003). Thus, it is possible that selection on blooming time coincided with a pollinator shift.

Evidence suggests that shifts either to or from bumblebee pollination have occurred within the Himalayan clade of *Roscoea*. Bumblebees, while active in the environment, never land at flowers of *R. alpina* (Paudel et al. 2017), another species in the Himalayan clade (Ngamriabsakul et al. 2000), and are not visitors to either *R. purpurea* (Paudel et al. 2015; present results) or *R. ganeshensis* (unpubl. data). In contrast, bumblebees are the effective visitors to *R. tumjensis* (present results) and contribute more than 90% of the pollination service to *R. auriculata*, *R. capitata*, and *R. tumjensis* (unpubl. data). The close phylogenetic relationships between *R. alpina* and *R. auriculata*, which are likely sister species, and between *R. tumjensis* and *R. ganeshensis*, which may be sisters (Ngamriabsakul et al. 2000) highlight the significance of these pollinator shifts.

The flowering phenology in our two focal species suggest that either *Bombus* pollination arose in the *R. tumjensis* lineage because it relieved pollinator limitation among early flowering individuals or populations of an ancestral species that had a flowering phenology more like that of *R. purpurea*, or that pollination by *Philoliche longirostris* was adopted in the incipient *R. purpurea* lineage by late flowering plants of an ancestor with a phenology like that of *R. tumjensis*. Interaction with the novel pollinator, either bumblebee or tabanid fly, could then have favored a shift in flowering phenology to coincide with periods of greater activity of the new pollinator. This hypothesis requires critical examination, but, if correct, points to a potential for reciprocal selection on flowering phenology and pollinator behavioral fidelity in the evolutionary history of the newly isolated lineage.

Further work, including phylogenetic reconstruction with better resolution, could help establish the likely direction of divergence for either *R. purpurea* or *R. tumjensis*. Some pollination information is available for a more distantly related Indo-Chinese member of the genus, *R. schneideriana*, which is never visited by

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Bombus despite their active foraging at flowers of nearby plants (Zhang and Li 2008, Zhang, pers. comm.). Furthermore, the contrast in *Bombus* interactions with *R. capitata* and *R. ganeshensis*, each of which seems, from current geography, to have arisen in sympatry with *R. purpurea*, would provide independent instances of the evolution of isolating barriers. Comparison among these barriers and their sequential effects may shed additional light on the role of the seasonality of pollinators and their behavioral preferences and abilities in promoting species divergence in this group.

Overall, we found that temporal isolation and floral isolation are sufficient barriers to conserve species integrity of these alpine gingers, despite morphological similarity sufficient to confuse human observers (Cowley and Wilford 1998). This finding contributes to understanding the key role of flowering phenology and floral traits to maintain species integrity of closely related plant species in sympatry. Extending our findings to the genus as a whole, it seems likely that floral phenology and pollinator isolation have been major factors of speciation and diversification of *Roscoea*, particularly in the Himalayan clade. Extrinsic, ecological mating barriers seem to play the primary role in initiating lineage divergence in plants (Hodges and Arnold 1994; Husband and Sabara 2003; Baack et al. 2015), while intrinsic, postzygotic isolation accumulates with time since separation (Christie and Strauss 2018). *Roscoea purpurea* and *R. tumjensis* seem to present a particularly striking case of coordinated divergence in both phenology and pollinator identity, one that could have arisen rapidly. We have identified a potential selective advantage underlying such divergence: escape from pollen limitation at the phenological margins of a breeding season by adopting a novel pollinator with a novel seasonal abundance. Given the prevalence of pollen limitation among flowering plants (Knight et al. 2005), the potential for such rapid emergence of mating barriers must be common in plant populations, particularly in the regions where topography or other geographical features create strong gradients in seasonality over short distances.

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AUTHOR CONTRIBUTIONS

B.R.P., M.B., M.S., A.G.D., and Q.J.L. designed the experiment, B.R.P. conducted field experiment, B.R.P., M.B., M.S., A.G.D., and Q.J.L. analyzed the data, wrote the manuscript, which was reviewed by all authors.

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