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Reproductive ecology of medicinally important Kashmir Himalayan species of *Digitalis* L.

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Abstract

Reproductive biology, encompassing phenology, floral biology, pollination and breeding systems, was investigated in three pharmaceutically important and anthropogenically exploited species of *Digitalis* in the montane habitats of the Kashmir Himalaya. Flowers in all three species (*Digitalis purpurea*, *Digitalis grandiflora* and *Digitalis lanata*) are aggregated in racemes. Corollas are characteristically bell shaped enclosing four, didynamously arranged stamens. The stigma is deeply bifid only in *D. purpurea*. The three species over-winter as underground tubers and sprouting during the following spring occurs first in *D. lanata*, followed by *D. purpurea* and *D. grandiflora*. Floral bud opening in an inflorescence in all three species occurs sequentially from the bottom upwards in an acropetal succession and male and female phases are temporally separated with basal flowers entering the male phase first followed by the upper flowers in a regular sequence. Only after shedding the pollen grains completely do the flowers shift to the female phase. Resource allocation in all three species is more towards maleness than femaleness during anthesis. The species are principally insect pollinated and selfing is denied because of protandry and spatial separation of the stigma and anthers. Copious amounts of viable pollen grains produced by these species aid in successful pollination, fertilization and seed production. Studies revealed that none of these species operates autogamy because hand-pollinated flowers with self-pollen or bagged flowers (foreign pollen excluded) failed to set seed. High seed output in open-pollinated flowers and high pollen–ovule ratios also indicate the outbreeding nature of these species. Thus, any reduction in the number of individuals constituting a population would significantly reduce the floral display and consequent rewards to the pollinators, with a subsequent influence on seed production. Hence, data on the reproductive biology of overexploited species, such as *Digitalis* spp., is of pivotal importance in formulating effective conservation strategies.

Keywords: breeding system, *Digitalis*, phenology, pollen–ovule ratio, pollination mechanism.

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Introduction

Reproductive ecology is of fundamental importance in determining the barriers to production of reproductive propagules and is of pivotal significance in formulating strategies for the conservation of taxa (Gross *et al*. 2003; Tandon *et al*. 2003), particularly with small population sizes (Ohara *et al*. 2006). Because of unprecedented increases in anthropogenic causes of population decline, conservation biologists have extensively studied the effects of small population size on demography (Menges 1991), reproductive success (Young & Brown 1998) and genetic variation (Prober & Brown 1994). Although several taxa in the family Scrophulariaceae, such as *Mimulus guttatus* (Dole 1992), *Verbascum thapsus* (Donnelly *et al*. 1998), *Antirrhinum* sp. (Jones & Reithel 2001), *Veronica* sp. (Kampny & Dengler 1997) and *Scrophularia* sp. (Ortega & Devesa 1993), have been investigated with respect to different aspects of their reproductive biology, very limited information exists on the floral biology, pollination mechanisms, breeding systems and reproductive output of *Digitalis* (Sletvold 2002, 2005).

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The genus *Digitalis* L. belongs to the family Scrophulariaceae and is represented in the Kashmir Himalaya by three species, namely, *Digitalis purpurea* L. *Digitalis grandiflora* Mill and *Digitalis lanata* Ehrh. These species are sources of pharmaceutically important cardiac glycosides, such as digitalin and digoxin, which are used as myocardial stimulants. In addition, tannins, inositol, luteolin and fatty acids, such as myristic, palmitic, oleic, linoleic and linolenic acids, are also present in different plant parts. This medicinal potential of the taxa has led to over exploitation, which has resulted in reductions in the number and size of their populations over the entire Kashmir Himalayan region (Nazir 2004). In view of the significance of reproductive ecology studies in the management and conservation of such taxa, the present study examined in detail the floral biology, phenology, floral visitation and breeding systems operating in the three species of *Digitalis* that occur in Kashmir Himalaya.

Materials and methods

Study sites

The Valley of Kashmir, situated in the northern fringe of the Indian subcontinent between 33°22′ and 34°50′N latitudes and between 73°55′ and 75°33′E longitudes, covering an area of approximately 16 000 km^2, is formed by a girdling chain of Himalayan mountains, namely the Pir Panjal in the south and the Great Himalayan range in the south-east to north-east and the west. The climate of the Valley is predominantly temperate, changing to subalpine and alpine higher up in the mountains. The present study was carried out on naturally occurring populations of *D. lanata* in the Ferozpur area and *D. purpurea* and *D. grandiflora* in the Gulmarg area of the Kashmir Himalaya, at an altitude of 2150 and 2650 m a.s.l., respectively. The study area lies at a distance of approximately 55 km to the west of Srinagar city and is an unprotected area under considerable anthropogenic stress because of grazing and trampling.

Phenology and floral biology

For phenological observations 10 randomly selected healthy individuals were tagged in populations of the three species and were inspected throughout the growing season to record the onset and duration of various phenological events from sprouting to senescence.

Breeding system

Studies on the breeding system included analyses of the following aspects.

Anthesis and stigma receptivity On each of 10 plants, three flowers from an inflorescence were collected on day 0 to day 8 of anthesis because after 8 days most flowers had senesced. Observations on anthesis and stigma receptivity were made at different developmental stages. The time taken by individual flowers and whole inflorescences to bloom was recorded. Stigma receptivity was checked by fixing stigmas of different ages in Carnoy's fixative for 1 h, followed by staining in a mixture of 2 mL 1% aqueous acid fuchsin, 2 mL 1% aqueous light green, 40 mL lactic acid and 46 mL distilled water (Lewis 1979). The stained preparations, after mounting on glass slides, were observed under a microscope. Stigmas carrying germinating pollen grains on their surface were considered receptive. To estimate the duration of stigma receptivity, the stigmas were pollinated manually by dusting the pollen from freshly dehisced anthers from a plant approximately 5 m from the recipient plant onto stigmas of different ages with the help of sterilized needles and grease-free glass slides. These manually pollinated stigmas were kept in 1% KOH for 2–3 s, and then stained in a mixture of 1:1 cotton blue and lactophenol and examined periodically under a microscope. Data collected from these stigmas were recorded for determining the duration of stigma receptivity.

Pollen viability and pollen–ovule ratio Pollen stainability and viability were determined by squashing freshly dehisced anthers in 1% acetocarmine and 1% triphenyl tetrazolium chloride (TTC). The slides were examined using a light microscope. Counts of viable and non-viable pollen grains were made from randomly chosen fields of view at 10×. All deeply stained pollen grains in TTC preparations were considered viable (Shivanna & Rangaswamy 1992).

The pollen–ovule ratio (P/O) was calculated following Cruden's (1977) method using the following expression:

$$\mathrm{P/O} = \frac{\text{Pollen count per anther} \times \text{No. of anthers per flower}}{\text{No. of ovules per flower}}$$

The total number of pollen grains per anther was calculated by squashing an anther in 10 drops of water. After thorough shaking, the pollen count was made in several drops taken at random with the help of a pipette. The number of pollen grains per drop of pollen suspension was multiplied by the total number of water drops taken initially to obtain the total number of pollen grains per anther. The ovules were counted by dissecting the ovaries.

In-vitro pollen germination Following Brewbaker and Kwack (1963), an *in vitro* germination technique was used to study pollen germination and pollen tube growth. Active growth of pollen tubes under *in vitro* conditions

was generally obtained within 1–2 h of incubation at 22°C under dark conditions. The cultures, fixed by adding a drop of 10% ethanol, were scored in cotton blue stain under 10× and 40× magnification to estimate the percentage of pollen germination.

Pollination studies To check the incidence of wind pollination, glass slides smeared with glycerine were suspended from T-shaped stands fixed around the tagged plants for 24 h and studied under a microscope for species-specific pollen grains.

The frequency of insect visitations (number of times recorded during the observations) to the flowers was recorded on sunny and cloudy days. A total of 50 pollinator observation sessions over different times of the day (from 9.00 AM to 3.00 PM) were conducted either by one observer, or by two observers working in different areas. Each observation session was 30-min long, during which observers watched a 3 m^2 area. Each insect was followed as it entered the observation area and its actions and behavior were recorded. Representative floral visitors were trapped, anesthetized with NaCN and scrutinized for pollen load on their body parts. The behavior of visitors during the time of their visitation and the duration of their visit to a flower was recorded. The pollinators were identified at the Department of Zoology, University of Kashmir, Srinagar (J&K), India.

Breeding behavior Breeding behavior of the three species was studied using the following experiments:

1 Some unemasculated individual flowers/inflorescences were covered with butter paper bags to test for forced selfing.
2 To check for outcrossing ability unopened flower buds were emasculated and subjected to the following treatments.
    1 Flowers left open to cross pollinate.
    2 Flowers covered with bags and not allowed to pollinate to test for apomictic seed development.
    3 Receptive stigmas hand pollinated using pollen from flowers of the same plant to test for geitonogamy.
    4 Receptive stigmas of several flowers of the same species were impregnated with pollen from flowers borne on different individuals of the same species to test for xenogamy.

Resource allocation

To keep destructive sampling to a minimum, five randomly selected individuals from each population were harvested at the peak flowering stage. The harvested individuals were separated into stem, leaves, flowers and male and female reproductive parts. The plant material was oven-dried at 80°C for 24 h to a constant weight and the dry mass (which represented the amount of resources allocated) of each component was determined using an electronic balance.

Results

Floral morphology and display

Flowers in all three species are aggregated in racemes, but the pedicels are very short in *D. lanata*. The total depth of the floral tube, its dimensions, and the length of the style and stamens are given in Table 1. The flowers are smallest at the top of the flowering spikes in *D. purpurea* and *D. grandiflora*, but in *D. lanata* there is no diminution in corolla length or in the length of the long pair of stamens. Flowers of all three species are characterized by variously colored bell-shaped corollas.

The anthers and stigmas of *D. purpurea* and *D. grandiflora* are contained within the corolla bell. In *D. lanata*, however, the long stamens may protrude slightly above the corolla bell at the time of dehiscence. There are four stamens in all three species with long filiform, polyandrous filaments. The stigma is completely bifid in *D. purpurea*, while in *D. grandiflora* and *D. lanata* it is slightly bilobed. The color of the style also varies among the three species (Table 1).

Phenological behavior

Individuals of the three species over-winter in the form of underground tubers that remain dormant throughout the winter months. With the onset of the spring season, growth is resumed and the dormant tubers sprout into juvenile shoots; however, the time of sprouting varies between species. The *D. lanata* tubers are first to sprout (with initiation of rosette leaves) during mid March, while those of *D. purpurea* and *D. grandiflora* sprout from mid April (Fig. 1). Furthermore, the data are indicative of a highly variable protracted pattern of sprouting in the three species. Sprouting lasts for 21 days in *D. lanata* and for approximately 25 and 27 days in *D. grandiflora* and *D. purpurea*, respectively. Subsequent to the appearance of the above-ground rosette leaves, vegetative development begins rapidly, at which time the plants switch over to the sexual phase by producing floral buds.

Individuals of *D. lanata* are the first to initiate bud production from the month of May, while individuals of *D. purpurea* and *D. grandiflora* enter into the sexual phase approximately 6 weeks later (in June). The time taken from the initiation of floral bud production to the initiation of anthesis is similar in all three species and ranges from 44 to 49 days. Floral buds open in an acropetal order. The central axis, that is, the main spike, is the first to

Table 1 Floral attributes of three species of *Digitalis* taken at the stage of complete anther dehiscence, but before the opening of stigmas

Floral attribute	Name of the species		
	Digitalis purpurea	*Digitalis grandiflora*	*Digitalis lanata*
No. inflorescences per plant	5.1 ± 2.23	3.2 ± 1.22	6.1 ± 3.24
No. flowers per plant	53.3 ± 11.53	21.2 ± 6.28	27.2 ± 7.22
Flower color	Purple, white yellow or pink with black and white dots at the mouth of the flower, rarely creamish yellow	Yellowish, marked with brown spots	Creamy white with purple-brown mottle on the bell
Calyx	Long, ovate, toothed, lanceolate, acute, 1.4–1.2-cm long	Lobed, linear-lanceolate, hairy, acute, 0.8–0.6-cm long	Dense woolly, lobed, linear, 0.9–0.4-cm long
Corolla	Bell shaped and varying in length from 4.0 to 3.4 cm	Bell shaped and 3.1–2.7 cm long	Narrow base widening suddenly into a globular chamber with a strongly reflexed lip, approximately 1.3-cm long
Stamens	Four, creamish white with purple dots, didynamously arranged, filiform and polyandrous, long stamens 3.3–2.9 cm in length; short stamens 3.0–2.8 cm in length	Four, yellowish creamy, didynamously arranged, filiform and polyandrous, long stamens 2.7–2.4 cm in length; short stamens 2.2–2.0 cm in length	Four, yellowish, didynamously arranged, filiform and polyandrous, long stamens approx. 1.25 cm in length; short stamens 1.3–1.1 cm in length
Stigma	White, deeply bifid ranging in length from 3.8 to 3.5 cm	Creamish white, slightly bifid approximately 2.3 cm long	Creamish yellow, slightly bifid approximately 1.4 cm long

Mean ± standard deviation based on 10 replicates.

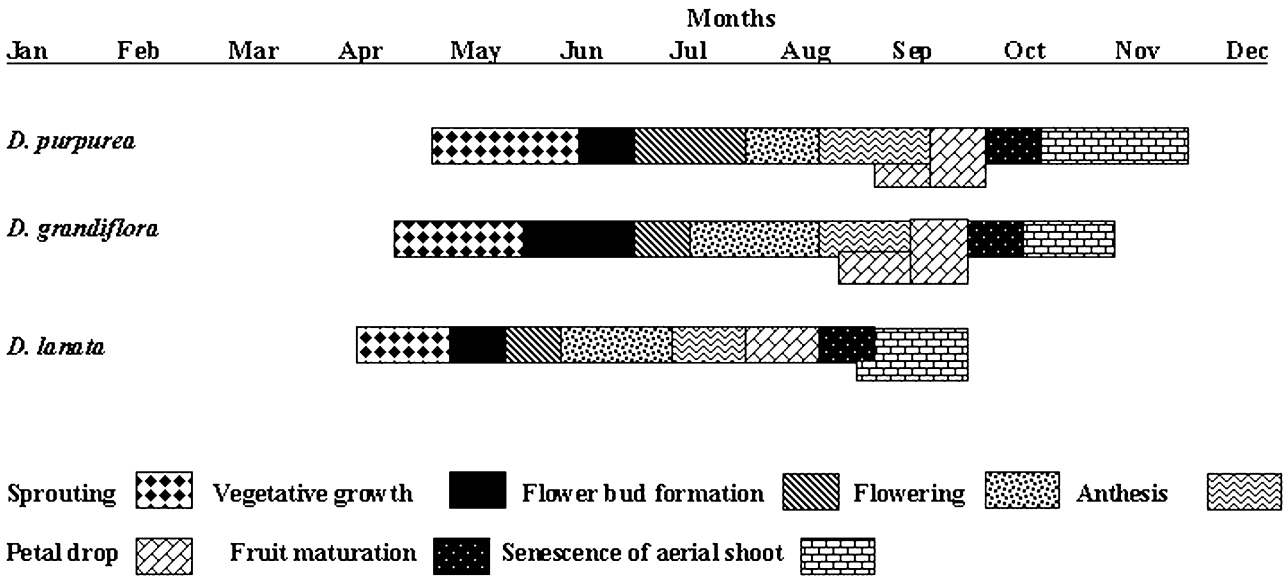

Fig. 1 Chronology of various phenophases in the three species of *Digitalis* based on 10 tagged plants.

flower and the lateral spikes start to bloom when the central spike is approximately two-thirds through its flowering. During the present investigation it was observed that flowering in *D. lanata* commences in May, in *D. grandiflora* flowering commences in early June, while in *D. purpurea* flowering starts in mid June. Usually it takes approximately 1–2 weeks for a plant to open all its flowers. In all three species, floral buds start blooming from June through to August; the process first begins in *D. lanata*, followed by *D. grandiflora* and then *D. purpurea*. This process continues for 15–19 days. After anthesis, the flowers remain open for 15–19 days. Later, petal shedding begins towards the end of June in *D. lanata*, followed by *D. grandiflora* in early August and *D. purpurea* by mid

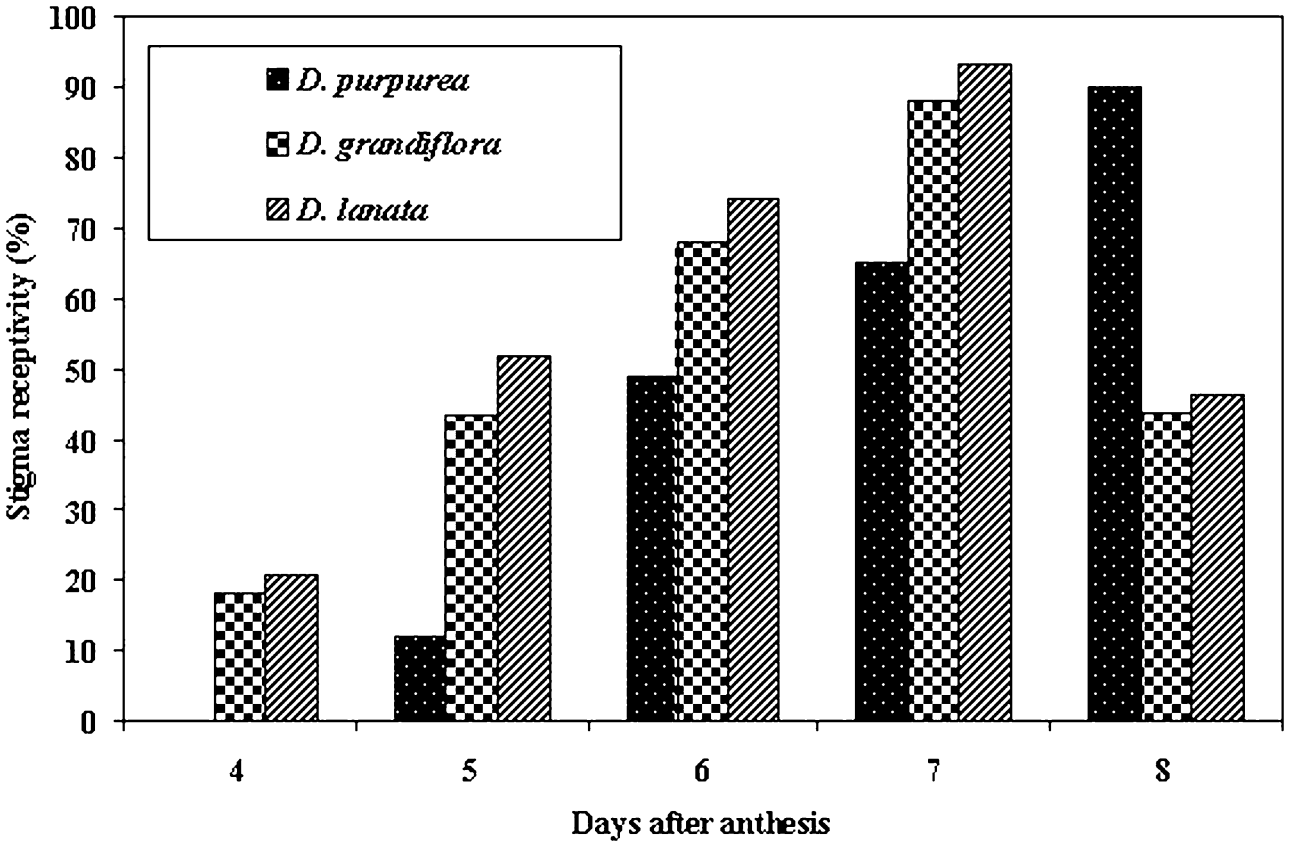

Fig. 2 Stigma receptivity in three Kashmir Himalayan species of *Digitalis* (percentage stigma receptivity based on 10 replicates).

August. It takes approximately 15–17 days for all three species to complete the petal-shedding stage (Fig. 1).

As all flowers of these species do not shed their petals simultaneously, it takes varying number of days for the fruits to mature fully. In *D. lanata* approximately 93% of fruits mature in the beginning of August, while in *D. grandiflora* and *D. purpurea*, fruit maturation happens towards the end of September.

Pollen presentation pattern

In *D. purpurea* and *D. grandiflora*, anthers of the long pair of the four didynamously arranged stamens dehisce first and then dehiscence occurs in the short pair. In *D. lanata*, the long pair of anthers move to the sides of the bell and the second pair elongates and dehisces in the top central position of the corolla bell. There is only a little lateral movement of the long pair of anthers in *D. purpurea* and *D. grandiflora*, and as such the anthers remain close to the ceiling of the corolla tube. In dull weather and under cool conditions pollen is shed over 4–6 days. However, on sunny days pollen presentation occurs for a reduced period of time.

Stigma opening and receptivity

Stigma opening in *D. purpurea* almost overlaps with pollen dispersal and occurs on the fifth day of anther dehiscence, that is, when the short pair of anthers undergo dehiscence. In *D. grandiflora* and *D. lanata* stigmas open on the fourth day of pollen dispersal. Stigmas in *D. purpurea* attain receptivity on the fifth day of anther dehiscence and receptivity increases to approximately 90% after 8 days of anther dehiscence. Likewise, in *D. grandiflora* and *D. lanata* stigmas become receptive on the fourth day of anther dehiscence and attain peak receptivity of approximately 88 and 93%, respectively, after 7 days of anther dehiscence (Fig. 2).

Nectar secretion

Nectar secretion begins 1 day before the first pair of anthers dehisce, although in negligible amounts. Secretion continues throughout the whole life of the corolla in all species. However, the peak secretion period (when appreciably higher amounts of nectar are available) coincides with peak anther dehiscence, thus, enhancing the chances of pollination by insect visitors.

Pollination system

The three species investigated in the present study are principally insect pollinated because their zygomorphic flower structure, particularly the position of the reproductive parts, bilipped corolla and protruded stigma are contrivances that promote insect pollination. Wind pollination is ruled out because glycerine-smeared hanging slides did not trap any pollen grains from these species. Furthermore, selfing is denied because the three species are protandrous and male and female sex organs are spatially separated with the stigma being placed ahead of the anthers.

Insect visitation and pollen load

Insect species that visited the flowers of the three species include *Apis mellifera, Apis cerana, Lasioglossum* sp., *Xylocopa valga, Anthidium manicatum, Bombus tunicatus* and *Bombus lucorum* (Table 2). Unlike *Lasioglossum* sp., which visited flowers from noon onwards, the other pollinators visited flowers throughout the day. *Anthidium* sp. was confined to *D. lanata* only and visited its flowers throughout the day with peak frequency during the afternoon. Among the five pollinators, *B. lucorum* was the most frequent pollinator of *D. purpurea*, particularly during and after anther dehiscence (Table 2). Although a similar but less frequent pattern was observed with respect to *Lasioglossum* sp. and *X. valga*, *Apis* spp. made frequent visits to flowers of *D. purpurea* mostly prior to anther dehiscence (Table 2). In *D. grandiflora* also, *B. lucorum* and *X. valga* were the most common visitors to flowers at later stages of their development. Unlike *D. purpurea* and *D. grandiflora*, the most frequent visitor to flowers of *D. lanata* was *B. tunicatus*. In addition, *Anthidium manictum* was seen visiting the flowers of *D. lanata* only (Table 2). The data reveal that *Bombus* spp. and *X. valga* made numerous visits to the flowers at the anthesis and post-anthesis stages in all three species and usually favored older flowers because the stigmas in these flowers opened during the peak period of nectar secretion, and by the time the stigmas are opening, the nectar in the tube rises to a height at which they can just reach it. *Apis* spp. visit the flowers and consume mostly pollen and rarely suck nectar. Individuals carry away the pollen deposited on the lower lip of the corolla. *Lasioglossum* sp. also forage pollen, but they do so directly from the anthers that remain concealed within the upper lip to which they cling. In this process the pollen falls on their head, thorax and abdomen. As the pollen-laden bees retreat from the flowers they brush against the protruding stigma and pollinate it. Individuals of *X. valga* and *Bombus* spp. display an interesting behavior of hovering around the inflorescence once they approach it and then alight on one of the flowers to penetrate their head deep inside the corolla tube, suck nectar and bring about pollination in the same way as *Apis* spp. do. The pollen load on different body parts of the pollinators is given in Table 3.

Mating system

The results of the experiments involving the different pollination treatments (autogamy, geitonogamy, xenogamy,

Table 2 Frequency of pollinator visits to the flowers of *Digitalis* species at different stages of anther development

Name of species	Insect	Proportion of total visits (%) Prior to anther dehiscence	During and after anther dehiscence with stigma closed	During and after anther dehiscence with stigma open
Digitalis purpurea	*Apis mellifera*	17.18	10.92	7.88
	Apis cerana	27.86	8.73	5.38
	Lasioglossum sp.	11.45	23.80	21.86
	Xylocopa valga	21.37	22.42	29.03
	Bombus lucorum	22.14	34.11	35.84
Digitalis grandiflora	*Apis mellifera*	12.00	19.77	10.79
	Apis cerana	24.00	9.88	6.12
	Lasioglossum sp.	28.80	22.88	12.59
	Xylocopa valga	15.20	19.49	34.89
	Bombus lucorum	20.00	27.97	35.60
Digitalis lanata	*Apis mellifera*	10.34	11.90	6.84
	Apis cerana	20.68	4.46	8.17
	Lasioglossum sp.	18.62	22.62	9.51
	Xylocopa valga	30.34	23.51	23.48
	Bombus tunicatus	20.00	28.87	26.75
	Anthidinm manicatum	0.00	8.63	25.26

Table 3 Number of pollen grains (pollen load) on various body parts of the insects visiting *Digitqlis* species

Name of species	Insect	Pollen load on body parts				
		Legs	Wings	Head	Thorax	Abdomen
Digitalis purpurea	*Apis mellifera*	14.00 ± 1.47	4.80 ± 0.001	8.50 ± 0.50	2.76 ± 0.04	1.11 ± 0.20
	Apis cerana	25.00 ± 10.04	3.40 ± 1.28	4.90 ± 0.02	2.39 ± 0.024	0.58 ± 0.015
	Lasioglossum sp.	4.50 ± 0.64	3.00 ± 0.70	3.2 ± 1.10	0.27 ± 0.013	2.27 ± 0.039
	Xylocopa valga	241.00 ± 80.85	13.25 ± 1.03	14.20 ± 1.08	4.03 ± 0.48	44.4 ± 12.50
	Bombus lucorum	356 ± 44.43	22.5 ± 3.66	26 ± 10.09	4.13 ± 0.04	691.13 ± 112.37
Digitalis grandiflora	*Apis mellifera*	–	1.96 ± 0.05	7.10 ± 1.30	5.80 ± 3.80	1.63 ± 0.002
	Apis cerana	41.38 ± 5.08	1.26 ± 0.041	2.20 ± 0.4	3.5 ± 1.4	1.42 ± 0.025
	Lasioglossum sp.	63.81 ± 3.80	2.54 ± 0.05	3.40 ± 1.10	8.50 ± 0.5	7.10 ± 1.30
	Xylocopa valga	82.55 ± 3.55	–	0.77 ± 0.40	–	1.84 ± 0.02
	Bombus lucorum	73.03 ± 2.52	20.30 ± 8.46	1.5 ± 0.02	0.29 ± 0.20	0.81 ± 0.02
Digitalis lanata	*Apis mellifera*	75.12 ± 10.8	2.27 ± 0.04	1.80 ± 0.19	1.84 ± 0.04	4.90 ± 0.002
	Apis cerana	58.96 ± 3.69	10.43 ± 3.60	2.2 ± 0.04	–	4.50 ± 0.64
	Lasioglossum sp.	6.76 ± 3.03	–	1.43 ± 0.60	0.31 ± 0.015	0.09 ± 0.0001
	Xylocopa valga	0.74 ± 0.001	–	–	–	–
	Bombus tunicatus	16 ± 0.79	1.5 ± 0.02	6.71 ± 0.09	1.42 ± 0.025	2.8 ± 0.33
	Anthidinm manicatum	94.16 ± 12.60	3.80 ± 1.35	1.90 ± 0.08	3.00 ± 0.71	3.90 ± 2.00

Mean ± standard deviation based on five replicates.

Table 4 Reproductive output in *Digitalis* species following different pollination treatments

Mode	Percentage seed set		
	D. purpurea	*D. grandiflora*	*D. lanata*
Forced selfing (autogamy)	0.00	0.00	0.00
Hand-pollination with self pollen (geitonogamy)	11.02	8.01	9.42
Hand-pollination with pollen of different plant (xenogamy)	66.40	59.75	51.25
Pollination prevented (apomixis)	0.00	0.00	0.00
Open pollination	81.95	76.29	79.80

apomictic behavior and open [natural] pollination) brought to light the dependence of the flowers of *Digitalis* species on biotic agents for pollen transfer; thus, these species are habitual outbreeders (Table 4). It was further observed that the flowers that were bagged, but not hand-pollinated, did not set seeds. However, geitonogamy, xenogamy and the open-pollination treatment showed significant differences in the percentage of fruit set. In the open–pollination treatment the percentage of seed set was significantly higher in all three species compared with flowers subjected to xenogamy by hand. None of the species exhibited apomictic behavior.

Pollen–ovule ratio

Estimates of the total number of pollen grains and ovules produced by a flower in these species were used to compute the pollen–ovule ratio (Table 5). The three species exhibited significant variations in the number of pollen grains per flower, although the anther number per flower was four in all three species. The average pollen production per flower was 53 980 ± 1353.64 in *D. purpurea*, 37 084.5 ± 587.34 in *D. grandiflora* and 30 105.2 ± 463.80 in *D. lanata*. The production of ovules per flower also varied considerably among the species, ranging from 454.4 ± 73.11 in *D. purpurea*, 397.0 ± 49.89 in *D. grandiflora* to 338.6 ± 51.48 in *D. lanata* (Table 5).

Pollen viability and in vitro *pollen germination*

Tabulated data (Table 6) reveal that the percentage of pollen viability in the three species is >90%, indicating that these species yield copious quantities of fertile male gametes that enhance the chances of pollination and fertilization and ensure successful seed formation, which is substantiated by the response of the pollen to *ex situ* germination.

Table 5 Pollen–ovule ratio in *Digitalis* species

Attribute	Name of the species *D. purpurea*	*D. grandiflora*	*D. lanata*
No. anthers/flower	04	04	04
No. pollen grains/anther	53 980 ± 1353.64	37 084.5 ± 587.34	30 105.2 ± 463.80
Total no. pollen grains/flower	215 920.0 ± 5414.56	148 338.0 ± 2349.36	120 420.8 ± 1855.20
No. ovules/flower	454.4 ± 73.11	397 ± 49.89	338.6 ± 51.48
Pollen–ovule ratio/flower	475.17 ± 18.51	373.64 ± 11.77	355.64 ± 9.00

Mean ± standard deviation based on 10 replicates.

Table 6 Pollen viability and germinability of *Digitalis* species

Attribute	*D. purpurea*	*D. grandiflora*	*D. lanata*
Mean no. viable pollen grains	4563.2 ± 1.48 (96.4)	3866.1 ± 0.74 (96.3)	4338.9 ± 0.81 (97.4)
Mean no. pollen grains that germinated	17.2 ± 7.99 (82.2)	18.1 ± 8.47 (86.0)	19.0 ± 9.06 (90.0)

Mean ± standard deviation based on five replicates. Figures in parentheses represent percentages.

Table 7 Resource allocation pattern in *Digitalis* species

Dry weight on a per plant basis (g)	*D. purpurea*	*D. grandiflora*	*D. lanata*
Stem	17.2 ± 1.30 (54.7)	15.6 ± 1.01 (62.15)	14.4 ± 0.48 (59.75)
Leaves	9.3 ± 3.45 (29.60)	7.2 ± 1.06 (28.68)	6.8 ± 2.19 (28.21)
Flowers	4.9 ± 2.27 (5.6)	2.3 ± 0.57 (9.16)	2.9 ± 1.19 (12.0)
Stamens	0.469 ± 0.0057 (1.49)	0.10 ± 0.004 (0.39)	0.10 ± 0.007 (0.41)
Carpels	0.16 ± 0.004 (0.50)	0.04 ± 0.001 (0.15)	0.05 ± 0.001 (0.20)

Mean ± standard deviation based on five replicates. Figures in parentheses represent percent allocation to different plant parts.

Resource allocation

It is evident from the data (Table 7) that partitioning of resources is not uniform among the different parts of a plant in the three species. However, maximum resources are allocated to the growth and development of the stem, followed by the leaves and flowers. Furthermore, the percentage allocation to the reproductive parts, that is, the reproductive effort, was more towards maleness in all three species than towards femaleness, which points towards their outbreeding nature.

Discussion

Flower and inflorescence morphology

Flowers of *D. lanata, D. grandiflora* and *D. purpurea* are hermaphrodite and herkogamous with tubular corollas with the style and stigma placed above the anthers. This type of floral morphology is known to occur in many other taxa of Scrophulariaceae (Tank *et al*. 2006). The three investigated species bear highly specialized showy flowers, aggregated in racemes, which together with flower size may affect both the attractiveness to pollinators (Conner & Rush 1996; Grindeland *et al*. 2005; Kudo & Harder 2005) and the efficiency of pollen transfer (Nilsson 1988; Schemske & Ågren 1995).

Shoot and flowering phenology

The investigated species depict considerable variability in their flowering phenology. The duration and occurrence of different phenophases is shaped by several biotic and abiotic factors and the length of the growing season

typically defines the time available for flower production and fruit maturation (Olsson & Ågren 2002). Sprouting in *D. lanata* is initiated in early March, while *D. purpurea* and *D. grandiflora* begin to sprout during the second and third week of April, respectively (Fig. 1). This is probably because of the occurrence of *D. lanata* at a relatively lower altitude (2150 m) compared with the other two species, which inhabit habitats at higher altitudes (2650 m). In addition, anthesis is fairly asynchronous between the species. Flowers in *D. lanata* anthesce first and hence shed their petals first, and fruit development and senescence in this species commences early. In comparison, anthesis in *D. purpurea* and *D. grandiflora* commences late, and the subsequent phases are also postponed. However, floral bud opening in all three species occurs sequentially from the bottom upwards in an acropetal succession in an inflorescence. Basal flowers of the inflorescence mature and anthesce first followed by the upper flowers in a regular sequence. As the basal flowers anthesce, they enter the male phase first. By the time the pollen grains are completely shed in these flowers and they shift to the female phase, the adjacent upper flowers anthesce and enter the male phase and subsequently shift to the female phase after pollen shedding is complete. This process proceeds sequentially from the base upwards in an inflorescence. Spatial and temporal separation (protandry) of the sexes within the flowers of the three *Digitalis* species is pronounced. This phenomenon is characteristic of many other angiosperm taxa and has also been reported in *D. purpurea* by Grindeland *et al*. (2005). The combination of acropetal flowering and protandry is reported to have a great bearing on the nature of the breeding system operative in a species. For instance, Faegri and Vander Pijl (1971), Wyatt (1982), Lloyd and Webb (1987) and Wafai *et al*. (2005) reported that such a behavior helps to limit self pollination within an inflorescence (geitonogamy) and also enhances outcross pollination.

Pollination mechanism

The mechanism of pollination and the mating systems operative in plants are influenced by floral morphology, phenology and inflorescence architecture (Wyatt 1982; Richards 1986; Harder & Barrett 1995). Flowers of the three species of *Digitalis* examined in the present study are foraged by different types of pollinators (Tables 2,3), including bees, which generally visit long-tubed flowers characteristic of Scrophulariaceae (Pennell 1935). The most common pollinators included species of *Bombus, Apis* and *X. valga*. *Bombus lucorum* was the preferred pollinator of the two sympatric species of *D. purpurea* and *D. grandiflora*, although several other pollinators also visited flowers of these two species. Although *B. tunicatus* visited flowers of *D. lanata* only, its other effective pollinators included *Apis* spp. and *Anthidinum manicatum* (Table 3). By and large these pollinators at first preferentially foraged the lower functionally female flowers, then progressively foraged the upper flowers and then, after neglecting the topmost flowers in an inflorescence, switched to the lower functionally female flowers on the next plant. Pyke (1974) attributed the neglect of the uppermost flowers in such inflorescences among angiosperms to their decreased nectar presentation. This unique foraging behavior of the bees is believed to maximize the degree of xenogamy because the bees forage unidirectionally upwards in an inflorescence from the basal functionally female flowers to the upper functionally male flowers. This may reduce geitonogamous interfloral pollen transfer in a given inflorescence. After visiting a few flowers of an inflorescence, the bee shifts to the basal nectar-rich, functionally female flowers of the next plant, thus accomplishing the task of plant-to-plant pollen transfer. The same foraging behavior has also been reported in many other angiosperm taxa, including *Aconitum nepellus* (Heinrich 1979), *Aconitum heterophyllum* (Siddique 1991; Wafai *et al*. 2005), *Delphinium* sp. (Epling & Lewis 1952; Benham 1969; Cruden 1977; Wadington 1981) and *Digitalis* sp. (Faegri & Vander Pijl 1971; Wadington 1981; Richards 1986), which have protandrous, acropetally maturing flowers in much the same way as the species of *Digitalis* investigated in the present study.

Breeding system

To test the dependence of these species on insect pollinators, all three *Digitalis* species were studied using bagging experiments. Flowers hand pollinated with self-pollen or bagged to exclude foreign pollen failed to set seed, which points to the fact that none of these species operates autogamy. On the contrary, flowers allowed to open pollinate set 81.95, 76.29 and 79.80% seed, respectively, in *D. purpurea*, *D. grandiflora* and *D. lanata*, and those hand pollinated with foreign pollen set 66.40, 59.75 and 51.25% seeds, respectively (Table 4). Both these manipulations support the outbreeding nature of these species and production of seed by exclusive cross pollen. The lesser seed set in response to hand pollination is probably attributable to the deposition of much larger quantities of pollen on the stigma than optimally required for efficient pollen germination under natural conditions, which may lead to competitive inhibition of pollen germination and/or pollen tube growth further down the style. It is also quite probable that peak stigma receptivity may be missed during controlled pollination. Even the bagging of the flowers or inflorescences may affect seed set because in no way can the ideal conditions for optimal pollen landing and subsequent pollen germination be simulated through controlled pollinations. The open-pollinated flowers left

unbagged have multiple visits by pollinators protracted over a longer time period and consequently the chances of the pollen landing on the stigma during its peak receptivity are increased, which in turn may be responsible for the higher percentage of seed set. Experimental support for such a response has also been obtained by Young and Young (1992) and Maloof (2000) in *Corydalis caceana*. However, the operation of a mixed-mating system in many taxa of Scrophulariaceae, such as *Ourisia* sp., *Collinsia verna* and *Euphrasia willkommii* (Arroyo & Penazola 1990; Dole 1992; Ortega & Devesa 1993; Kalisz *et al*. 1999; Gomez 2002), is also on record.

Pollen–ovule ratio

An increase in the P/O ratio from autogamous to allogamous taxa (Cruden 1977; Spira 1980; Wyatt 1984; Crawley 1986; Preston 1986) provides a reasonable index of the nature of the breeding system operative in a species. In the presently investigated species of *Digitalis,* the P/O ratios of 475.17 ± 18.51 for *D. purpurea*, 373.64 ± 11.77 for *D. grandiflora* and 355.64 ± 9.00 for *D. lanata* (Table 5) are in tune with those of other facultative outcrossing taxa. The P/O ratios of these *Digitalis* species are not as high as those of obligate outcrossing species (Cruden 1977).

The species exhibited optimal pollen germination (Table 6), which clearly suggests that the pollen displayed by these species is highly fertile as deduced on similar grounds by Alexander (1969, 1980) and Shivanna and Rangaswamy (1992) in many other angiosperm taxa. The viable nature of the pollen was also shown by the acetocarmine, cotton blue and tetrazolium tests and *in vitro* pollen germination studies (Shivanna & Rangaswamy 1992).

Resource allocation pattern

An examination of the biomass allocation pattern in the species investigated in the present study reveals that the partitioning of resources is not uniform between reproductive (e.g. flowers) and vegetative (e.g. leaves and stems) functions. In addition, subdivision of reproductive resources among floral parts (e.g. stamens and carpels) is also variable, with more allocation towards male than female reproductive structures (Table 7). Such hierarchies in the allocation of biomass to somatic growth and different structures associated with reproduction are known to profoundly affect life-history traits (Worley *et al*. 2003; Tomimatsu & Ohara 2006). Increased allocation to male reproductive structures in the three species of *Digitalis* is consistent with the prediction of sex-allocation models (Charlesworth & Charlesworth 1987; Sakai 1993) that have pointed out more allocation to male reproductive organs and also to attractive floral structures, such as showy corollas in outcrossing species (Tomimatsu & Ohara 2006). Protandry, which was observed in the three species, is also known to promote increased male allocation (Huang *et al*. 2004).

Conservation

The present observations on features of phenology, floral morphology and resource allocation pattern, and the pollination studies, which provided inferences into the breeding systems operative in the three species of *Digitalis* in the Kashmir Himalaya, India, point towards the outbreeding nature of the species with absolute dependence on pollinators for pollination and subsequent seed set. Models of pollinator foraging indicate that plant population density, by constraining the frequency and duration of pollinator visits, is likely to have a crucial role to play in pollination success, seed production and in the likely success of the species in the face of multiple intrinsic and extrinsic factors limiting the size of the populations. Any further habitat destruction would further fragment the already sparse populations of the three species of *Digitalis*, which in turn would trigger a cascade of factors, including low availability of pollinators, loss of genetic diversity, altered age structure of populations, low seed production and thereby low seedling recruitment and reduced population viability. Thus, urgent measures need to be undertaken to restore the habitats of *Digitalis* species in the Kashmir Himalaya and such an approach will have a salutary effect on the demographic and reproductive attributes of their remaining populations.

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