Variation in *Impatiens* species in Northern Western Ghats: Role of reproductive ecology and spatial distribution

A thesis submitted towards partial fulfillment of B.S.-M.S. Dual Degree Programme

By

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Under the guidance of

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Certificate

This is to certify that this dissertation entitled 'Variation in *Impatiens* species in Northern Western Ghats: Role of reproductive ecology and spatial distribution' towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by 'Mihir Umarani' at IISER Pune under the supervision of 'Dr. Deepak Barua', Assistant Professor, Biology Division, IISER Pune during the academic year 2012-2013.

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Declaration

I hereby declare that the matter embodied in the thesis entitled ‘Variation in *Impatiens* species in Northern Western Ghats: Role of reproductive ecology and spatial distribution**’** are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of Dr. Deepak Barua, Assistant Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.

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Abstract

Variation, genetic or morphological, in populations of species is affected by many ecological parameters. In plant species, typically, population size, density, reproduction strategy, pollination and seed dispersal mechanisms, phenology and other life history traits are some of the major parameters. Rock outcrops provide a good system of isolated 'terrestrial islands' to assess the interactions of these parameters with the population dynamics. Diverse species of *Impatiens* with different backgrounds of endemism and habitat specificity prove to be ideal for comparisons of inter-intra population variation to detect the divergence of populations. Various ecological parameters were assessed for different populations of five *Impatiens* species and morphological variation was measured. Important result from this study is that, *I. lawii* populations show dramatic differences in their reproductive traits than other species. Primary reason for this result was a restricted gene flow which could have caused by ecological determinants such as narrow geographic distribution, habitat specificity and different levels of flower visitation for different populations.

List of figures

List of tables

Introduction

Spatial and genetic structures of plant populations result from multitude of processes such as mutations, selections, drifts and environmental heterogeneity and selection processes arising from them, however all these processes occur in the background of general biology of each plant species and their mechanisms of interacting with the environment. Therefore ecology of the species plays an important role of providing a platform on which selection and other processes can work and thus can help in predicting the spatial and temporal features of genetic variations in the populations. Many studies have been performed to assess the roles of ecological parameters determining the genetic structure i.e. spatial and temporal distribution of genotypes in the populations which can include the parameters like allele distribution in the populations or gene flow etc. Many ecological parameters such as population structures, life history, reproduction and dispersal mechanisms are known to have affected the genetic structure of the populations of plant species. (Hamrick and Godt, 1996)

To study these effects, one clear prerequisite is to have well defined populations which can be compared or studied together for assessing genetic variation or gene flow between the populations. Islands provide an ideal system to study the population dynamics both within and between populations. Rock outcrops provide an excellent alternative to the islands system. Rock outcrops are defined as “portions of exposed bedrock protruding above the soil level due to geological activities. The term includes landforms ranging from cliffs, isolated hills and platforms with diverse geology” (Watve, 2007).They exhibit highly specific environmental and habitat condition. Rock outcrops form very different microenvironment from their surrounding landscapes which make them fairly isolated habitats or ‘terrestrial islands’. Inselbergs, one form of rock outcrops are known to restrict the gene flow and carve out the genetic structure of populations. (Barbara et. al., 2009) Thus rocky outcrops can provide a good system to study the divergence process and its relation with the ecological factors on both inter- and intra-species level. (Watve, 2007; Porembski et. al., 1996; Lekhak and Yadav, 2012) Extreme environmental conditions make these outcrops a home to various highly endemic and specialized species. Such species naturally restricting to their specific environmental conditions, can form isolated populations. These characteristics make them good candidates to study the effect of ecological parameters on their gene flow.

Table 1: Summary of factors that can individually affect the genetic variation of populations and their predicted effects. (Hamrick and Loveless, 1984)

Trait	High inter-population variation	Low inter-population variation
Breeding System	Inbreeding	Outcrossing
Flower morphology	Hermaphroditic	Monoecious/dioecious
Pollination	Small bees	Large bees, bird/bats
Flowering Phenology	Asychronous	Synchronous
Time of reproduction	Monocarps	Polycarps
Life cycle	Annual	Long-lived
Geographic range	Regional/endemic	NC

NC: No clear correlation.

Floral morphology, breeding system, mode of reproduction, pollination and seed dispersal are some of the most important and observable traits which govern the genetic structure.

Effects of ecological determinants such as population structures, phonology, reproduction and dispersal mechanisms on within and between population genetic variation are well studied (Hamrick and Loveless,1984; Awadalla, et. al., 1997; Friedman et. al.2009; Hamrick et. al., 1996; Heywood et. al. 1996; Hutchinson and Templeton. 1999; Ibrahim et. al.1996; Schoen et. al. 1991; Schoen et. al.1996;Sork et. al. 1999; Ward et. al. 2005)

These parameters are correlated to varying degrees. Life cycle and dispersal modes can represent many of the other ecological variables and used suitably for assessing genetic structure.

Other factors that can potentially affect the genetic variation levels are geographical parameters and historical parameters i.e. distances between the populations or the establishment of the populations etc. Genetic variation would depend on geographic range of the population and spatial structure of the population and distances between populations. (Hutchinson and Templeton, 1999)

Some of the above listed parameters could become the criteria for choosing appropriate species for the study of effects of ecological parameters on variation within and between populations. Community level study has been performed on rock outcrops in north Western Ghats. (Watve, 2007) Some of the plant species found on these outcrops can form a good system to study the effects of ecological parameters on variations within and between populations. Among the plant community, species from *Impatiens* genera provide a good system to compare the effects of spatial structures and other geographical properties of populations on the dynamics of species at population level. . *I. lawii, I. balsamina, I. tomentosa, I. oppsitifolia, I. dalzellii, I. acaulis, I. pulcherrima* are some of the *Impatiens* species which exist on the highlands of northern parts of Western Ghats. These species show various levels of endemism and habitat specificity.

Other ecological and biological parameters can be assessed for these species and compared in the background of variety of levels of endemism and habitat specificities,

Five of these species were chosen as they provide a wide range of geographical distribution and variations in the habitats they are found in. All of these species are annual, monocarpic and have an explosive dispersal mode. Previous surveys (Sharma et. al. 1960, Dessai and Janardhanam, 1988; Lekhak and Yadav, 2012) provide some information about their geographic distribution, abundance, and habitat association.

Table 2: Summary of geographic distributions for five *Impatiens* species. (Flora of Maharashtra vol. 2; Watve, 2006; Dessai and Janardhanam, 1988; Lekhak and Yadav, 2012)

Species	**Geographic distribution**
Impatiens lawii	Narrowly endemic
Impatiens oppositifolia	Widespread
Impatiens dalzellii	Narrowly endemic
Impatiens tomentosa	Widespread
Impatiens balsamina	Widespread

The study of morphological variation was performed as an initial estimate of the variation between the populations of five of the above listed *Impatiens* species on three lateritic plateaus which were isolated from each other in varying degrees. Considering this aim, following objectives were set: 1) to assess and validate the information about spatial distribution of the five *Impatiens* species: *I. lawii, I. balsamina, I. tomentosa, I. dalzellii, I. oppositifolia*, which would be determined on three different spatial scales: Global, regional and on-habitat level. 2) To collect information of parameters related to reproductive ecology of these species: phenology, pollination biology, population parameters such as density and size. 3) Study traits for which variation between plateaus would be assessed: Vegetative traits such as number of leaves, leaf morphology, height variation, branching patterns as well as reproductive traits such as flower morphometry along with flowering, fruiting, and seed count of individuals. Traits that are related to reproductive biology can provide vital information about the amount of variation present specifically in reproductive traits which is an important part of total variation as it can be crucial in the species dynamics. Relations between possible causal factors such as phenology, reproductive ecology, spatial distributions, population density, flower-visiting species and variations between populations of these five species were studied.

Methods:

<u>Characterization of study sites</u>: Initial surveys of presence of chosen *Impatiens* were done in Kas, Chalkewadi, Thoseghar Mahabaleshwar, Panchgani, Mhavshi, Sada-waghapur.. Presence of more than two species of *Impatiens* was the first criterion for the selection of plateaus. Besides, factors such as easy accessibility and presence of rocky outcrop habitats were considered and three plateaus were chosen for the study: Kas, Chalkewadi and Thoseghar.

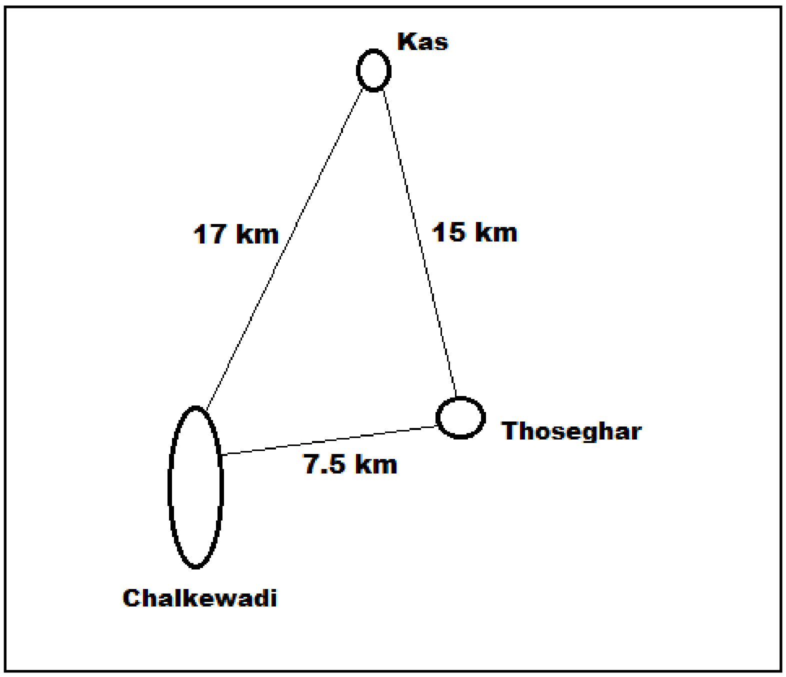

Fig. 1: Schematic diagram showing the relative sizes of 3 plateaus (Kas, Thoseghar, Chalkewadi) presented by sizes of ovals and aerial distances between them.

Description of plateaus: Kas ($17^0$43' N 73^0 49' E) plateau contains 2 table lands separated by two small valleys. The total area is approximately 3 sq.kms. The plateau is separated from surroundings by sharp slopes and visually distinguishable forest cover. Thoseghar ($17^0$34' N $73^0$54' E) is narrow strip of hill top stretching for about 5 kms. The total area is approximately 3sq.km. Chalkewadi: ($17^0$33' N $73^0$50' E) is the largest plateau and is approximately 8-9 kms long in the north-south direction. The plateau has less defined boundaries. The west boundary adjoins Koyna Wild Life Sanctuary. Total area is approximately 8-10sq. km.

Phenology: The following traits were recorded for five species of *Impatiens* on three plateaus: Height, Number of lateral branch number of leaves, senescent leaves, number of buds, number of flowers, flower color (only for *I. lawii*), number of total fruits, number of mature fruits, average number of seeds(mean of three fruits), number of dispersed fruits.

For each species on each plateau, three clusters (aggregated groups of individuals) were chosen where one cluster was larger and other two were smaller in comparison. 10-15 individual plants were chosen from the cluster for trait measurement. For *I. balsamina*, three clusters were chosen along the road (where they are most abundant). *I. dalzellii*, is rarely found in aggregations and for this species 10-15 independent individuals were used for measurement of traits when clusters could not be identified.

The observations were made for a period of six weeks between 5 September and 21 October. For the species on Kas plateau including *I. balsamina* population on the access road to Kas, observations were made on 7 September, 17 September, 27 September, 5 October and 12 October. For Chalkewadi and Thoseghar, the dates were 8 September, 18 September, 28 September, 6 October and 21 October.

Analysis: The timing for peak flowering (maximum number of open flowers/branch) was assessed for every species on each plateau. To estimate the duration of peak flowering we used the date where flowering was greater than or equal to 50% of the maximum flowering. If at the first sampling date, flower abundance was more than 50 % of the peak, this was assumed to be the start time. Similarly, we used the date when flowering dropped below 50% of the maximum flowering as the stop date for peak flowering. Flowering durations were compared for all the species and the plateaus. Peak timings of budding and fruiting were monitored as the date of maximum number of buds/branch and fruits/branch.

Pollinator survey: Pollinator surveys were conducted on two plateaus - Kas and Chalkewadi, to examine differences in the number and diversity of pollinators.

Surveys were performed for the one day for all the *Impatiens* species found on a plateau. On Chalkewadi, four clusters of *I. lawii* and *I. tomentosa*, and three clusters of *I. balsamina* were chosen. Observations were made on three different times during the day (7 am-9 am, 10 am -12 am, and 4 am-6am). Each cluster of *I. lawii* was observed for 10 minutes while the clusters of *I. tomentosa* and *I. balsamina* were observed for five minutes. When the clusters were observed for 10 minutes, the values were divided by 2 to intrapolate the result for 5 minutes.

For each species, clusters for each species were chosen to have around 50 flowers. (When more than 50 flowers were observed, visitation indices were standardized for 50 flowers by dividing the number of flowers observed and multiplying by 50) Other flowering species within the cluster, the surrounding habitat, general weather conditions were noted before each survey. Insect visitors to flowers were categorized into bees, moths/butterflies, ants, beetles and others, and the numbers of visitors were noted. Number of flowers visited by an individual of visiting species was noted in terms of three discreet categories (few, medium, many) and the mean time spent per flower was also recorded.

Analysis: The primary aim from this exercise was to compare species visits (qualitative and quantitative) for all impatiens species and the two plateaus. Two parameters were examines as indicators of visitation levels for the species across the plateaus: number of visitors and the total time spent by visitors. Total visitation time was estimated as the product of the number of visitors, number of flowers visited and the time spent per flower for each species.

For further analysis: Differences in visitors between *I. lawii* and *I. tomentosa*, and *I. balsamina* were examined for Chalkewadi using an ANOVA (Statistica). Differences between species and plateaus were examined for *I. lawii* and *I. tomentosa* in Kas and Chalkewadi using a factorial ANOVA design.

Variation in traits: Traits were categorized as vegetative and reproductive traits. The following vegetative traits were measured for ten samples for each plateau each for *I. lawii* and *I. tomentosa*: leaf area, width and length, height of the plant, number of lateral braches, and the number of leaves per branches. Leaf traits, area, width and length of the largest leaf were measured for *I. lawii* and *I. tomentosa* for all the three plateaus. Photographs of leaves were taken with an appropriate scale, and the length, width and area calculated using ImageJ. Other vegetative traits were measured as mentioned above during the phenology observations. Preliminary analysis revealed that length, width and area were all highly correlated with each other, and for all further analysis we only considered leaf area.

These traits were analyzed using factorial ANOVA with plateaus and species as factors. Post-hoc tests were performed for pair wise differences between plateaus.

The following reproductive traits were measured: Flower morphometry, maximum number of flowers and fruits, number of seeds /fruit.

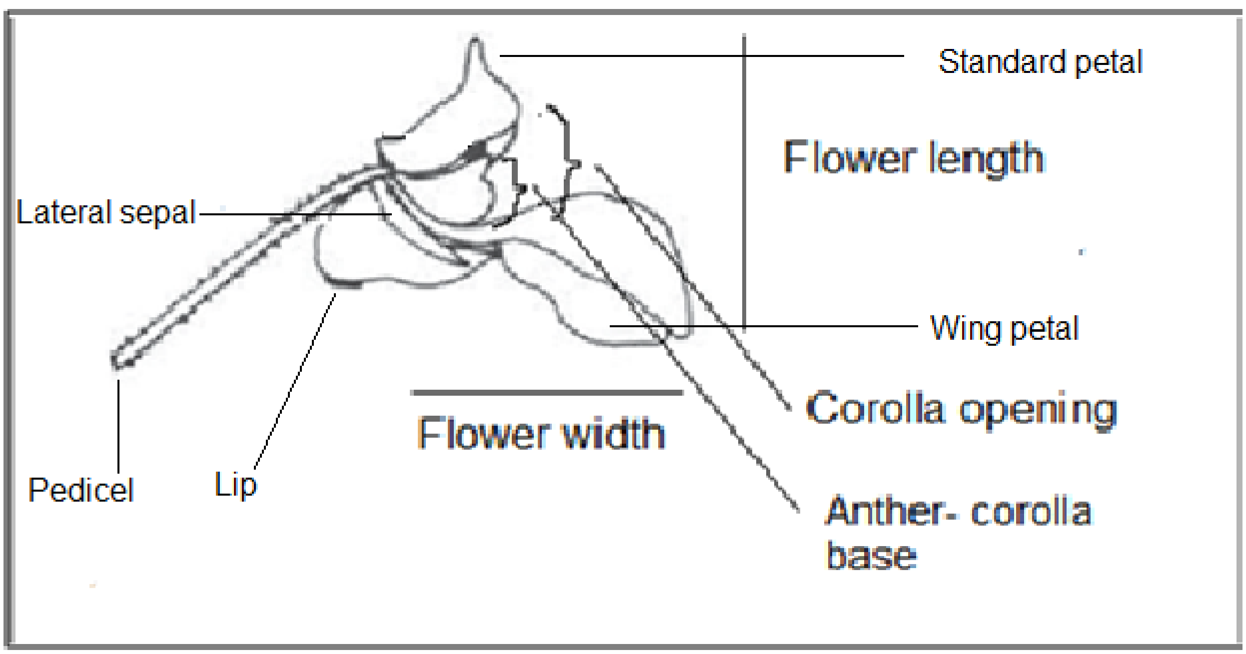

Fig.2 Diagrams of a typical *Impatiens* flower (resembling all the species of interest) with the labels for different flower parts. The image and naming system is adapted from (Dessai and Janardhanam, 2006)

For *I. lawii, I. dalzellii, I. oppositifolia* and *I. tomentosa*, ten flowers from different individuals were collected on each plateau. Flowers were plucked from the base of the pedicel and collected in wet tissues and measurements taken two-three hours later.

The following floral morphology traits were measured: Pedicel length, flower width (from pedicle tip to the end of face of the flower), flower height (the length spanned in the plane perpendicular to the one above, i.e. from the tip of standard petal to the bottom of lip part), length of sepals and petals, height and width of the corolla opening, length from anthers to corolla base, anther length, anther width, and length of Style.

Preliminary analysis revealed that flower height was highly correlated with other flower appendage related traits such as width, length of sepals, petals, lip depth and length. Therefore, only following traits were considered for the analysis: Pedicel length, flower length, standard petal length, corolla opening width, anther-corolla base distance, anther length, anther width and length of style. For these selected traits, factorial ANOVA was performed with plateaus and species as factors. Post-hoc analysis was used for each trait to assess pair wise differences. Tukey's HSD test was used for this analysis.

Other reproductive traits were derived from the phenology data.

All the other reproductive traits were analyzed in a similar way as the flower morphometry traits and all the populations were compared for each species. For the traits involving seed numbers, only the individuals with mature fruits i.e. non-zero number of seeds present were considered.

Line transects: Line transects was performed to examine habitat specificity of the species and to estimate various population size on Kas, Chalkewadi and Thoseghar. Transects were chosen from randomly taken coordinate points and direction from the map with the condition that it contained clusters of two *Impatiens* species. Geographical coordinated of the start and end point of transects were noted.

Three transects were performed for each plateaus. For Chalkewadi and Thoseghar plateaus, the length of all transect was 200 m. For Kas, transects were of 50m,100m and 200m long. Total area sampled for Chalkewadi was 4.6 sq.km. For each transect, data was taken in discreet steps of 1 sq. m. quadrant/plot. When *Impatiens* species were present, data was recorded every 2 m. In the absence of *Impatiens* species and when the vegetation visually appeared fairly homogenous, readings were taken every 5m. The quadrant was further divided into 16 equal squares.

For each quadrant/plot and within all 16 squares in the plot, the following factors were recorded: 1) Habitat type - categorized as vegetation, (presence of grass/shrubs) bare soil, bare rock, rocky crevices, water pool, stream, dry stream, road/other human related habitat; 2) Presence of the other species: These were – *Rotala* spp., *Cynotis* spp., *Murdannia* spp., *Pogostemon* spp., *Smithia* spp., *Eriocaulon* spp., *dipcadi* spp, grass, *Strobilanthus* spp., *Neanotis* spp., *Senecio* spp., *Utricularia* spp., *Rhampicarpa* spp, (other unknown species were categorized under 'other shrubs' and 'other herbs'); 3) Number of individuals of *I. lawii* and *I. tomentosa* present in the plot; 4) Maximum height of *I. lawii* and *I. tomentosa* present in the plot; 5) Soil depth in the center of the quadrant (taken by probing a steel rod into the soil and measuring height of underground part of the rod up to 30 cm). Depths greater than this were recorded as >30cms; 6) Slope - categorized as 0 to 3 (0 indicating flat land and 3 indicating a steep slope); 6) Maximum height of any individual of any species present in the plot; 7) Number of individuals of *I. lawii* and *I. tomentosa* inside each small square. (In Kas, *I. lawii* individuals were counted only in the diagonal squares and total number was extrapolated for whole quadrant/ plot) 7) Presence of *I. lawii/I. tomentosa* in the 2 m distance perpendicular to the direction of transect. 0, 1, 2 values were assigned referring to presence or absence of the species in the neighborhood. (1 if the species were present on any one side of a plot and 2 if the species were present on both the sides); 8) Presences/absence of Grassland/Shrubs/Karvi/Water body/Rocky patch/road in the 2 m distance perpendicular to the direction of transect. 0 indicates absence, 1 indicates presence of habitat on one side of a plot and 2 indicates the presence of habitat on both the sides.

Population estimates: Rough estimates for populations size of each species was calculated from information about habitat specificity of the species and estimates of extent of habitats derived from Google Earth images. For this estimation, association of *I. tomentosa* with shrubs was used to formulate population number which was derived as p = Area covered by Shrubs (measured from Google earth plus) * Density of *I. tomentosa* individuals within the shrubby areas (From correlation study)

For *I. lawii*, big clusters were previously recorded with their geographical coordinates: Total population was calculated by multiplying total area of clusters by average density of *I. lawii* individuals.

Multiple regression: To study the association of *Impatiens* species with habitats, multiple regression analysis was performed. Abundance of *I. tomentosa* and *I. lawii* was assessed in two forms: as number of individuals in a given quadrant and as presence/ absence of species.

Preliminary analysis of univariate regressions were undertaken to test for linearity of associations between habitat parameters and species. In all cases linear relationships showed the best fit, and hence multiple regressions which assume linear relationships were carried out.

Independent factors for this analysis were chosen from habitat types occurring inside or in the neighborhood of the plots. (Separate analyses for inside and outside habitats were performed) Dependant variables were chosen to be abundances of two *Impatiens* species. (Two forms: actual number of individuals and presence/absence information)

Multiple regression was also performed using abiotic factors as independent variables (soil depth, maximum vegetation height as a measure of shade presence and water presence)

RESULTS

All the five species were found to follow specific spatial patterns. One common criterion between their occurrences was that they were all found only at altitudes greater than 500 m. If the habitat types were categorized into three broad types: (top of the plateau, slope of the plateau, non-plateau areas*). I. lawii* occurred only on the top of plateaus*, I. dalzellii* occurred only on the slopes of the plateaus, *I. tomentosa* and *I. oppositifolia* were found both on the top of the plateau and on the slopes of the plateaus whereas *I. balsamina* showed very widespread occurrence majorly along the roads or human habitats in high elevations or slopes.

<u>Phenology</u>: Flowering times showed a lot of overlap between species and within species between plateaus (Fig. 3).

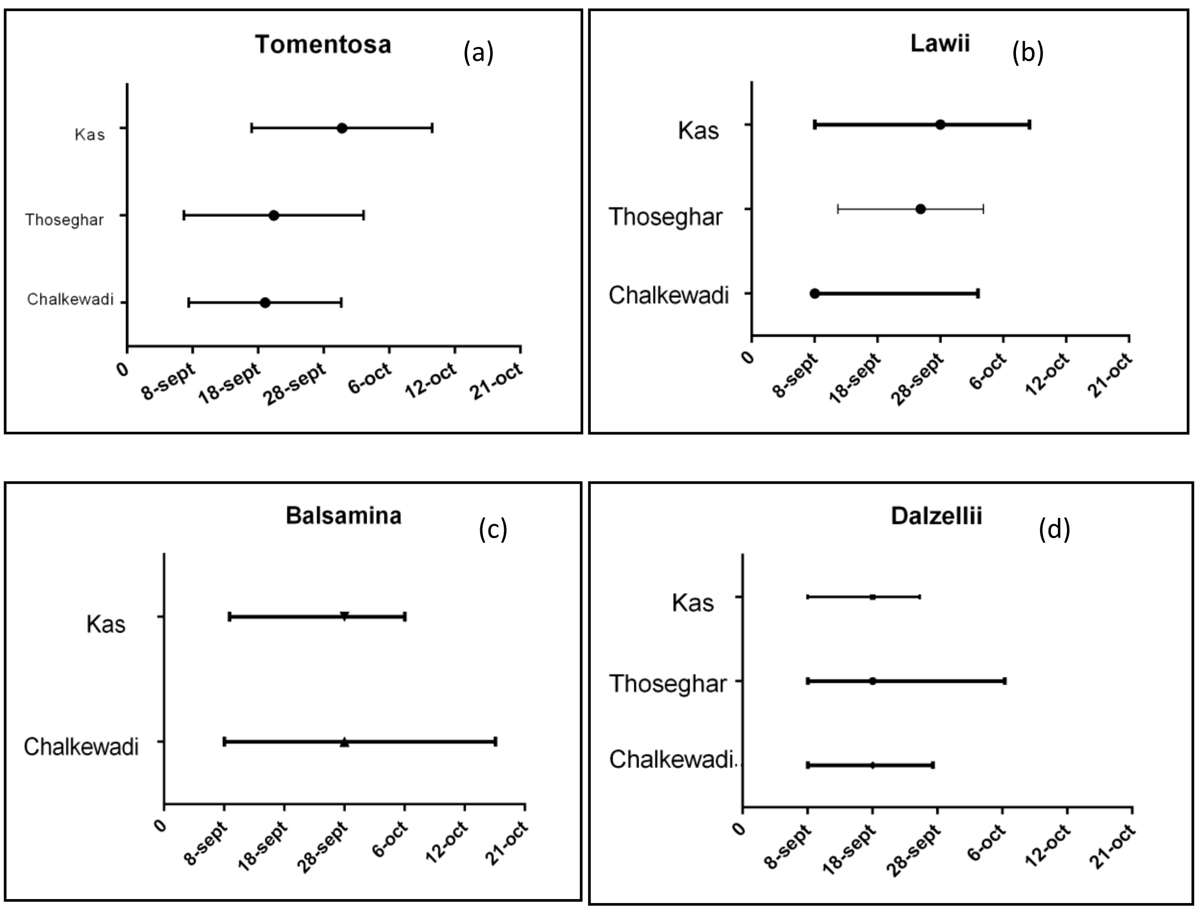

Fig. 3: Peak flowering duration (flowering greater than 50%) for (a*) I. tomentosa*, (b)*I. lawii*, (d) *I. dalzellii* on 3 plateaus (Kas, Thoseghar and Chalkewadi). (b) Flowering

For *I. lawii*, Chalkewadi populations showed early peak compared to other plateaus and then the flowering exhibited a gradual decline. The early flowering peak in *I. lawii* on Chalkewadi is interesting because there is not much difference in peak timings for the other species across plateaus.

Flowering peak timing for *I. balsamina* in both the locations (Kas and Chalkewadi) was similar. All *I. dalzellii* populations showed very early flowering peaks 17-18 September compared to the other species. *I. tomentosa* on Kas plateau showed a late peak (27th September) compared to the other plateaus.

Flowering durations were about 3-4 weeks for all the species except for *I. balsamina* in Chalkewadi, which lasted for 6 weeks.

In general, there was at least 2 weeks of time overlap between the plateaus for all the species which could allow enough gene exchange between populations.

Pollinator surveys: Floral visitors were similar on both the plateaus. On Kas, five species of bees, two species of flies, three different species of butterflies and moths, beetles and one species of ants were found to visit *Impatiens* flowers. On Chalkewadi, four species of bees, two species of butterflies and moths, beetles and one species of ants were observed to be visiting.

Table 3 provides summary of number of species observed visiting each population of two species on Kas and Chalkewadi. Bees and beetles appear to be the major visitors to flowers. Beetles show very different pattern of visitation from other visiting species. Beetles on entering a flower stay inside for a long time. We were unsure whether these were pollinators and thus they were analyzed separately and excluded from the later analysis. Pollination by bees and butterflies are well documented for *Impatiens* species in India. (Ramasubbu et.al., 2008,2011)

For species other than those of beetles, visitation time per flower varies typically from 2 to 10 seconds. For the bees, pollen deposited on the body was clearly visible. In this study, number of individuals visited and total visit time per flower per species are considered as estimates of pollinator visitation.

Table 3: Floral visitors for the *Impatiens* species. Observed visits are indicated by a letter Y. Negative sign and a letter in the parenthesis indicates absence on the specific plateau (C: Chalkewadi, K: Kas)

	I. lawii	***I. tomentosa***	***I. balsamina* (Only on Chalkewadi)**
Moths/butterflies	Y (-C)	Y	Y
Apis dorsata		Y	Y
Apis florea	Y (-C)	Y	Y
Bee1	Y (-C)	Y	Y
Fly 1	Y	Y	Y
Fly 2	Y	Y	Y
Ants	Y (-C)	Y	Y
Beetles	Y	Y (-C)	Y

For *I. balsamina*, all the bees were major visitors at both plateaus. For *I. lawii*, *A. florea* and fly 1 were major visitors at both plateaus. These species were generally smaller in size. For *I. tomentosa*, *A.dorsata*, butterflies and moths were major visitors. These species were comparably bigger species than others. *I. balsamina* individuals were observed only on Chalkewadi plateau, visitation by species for this species was significantly greater *than I. lawii* and *I. tomentosa*. On Chalkewadi plateau, visitation levels for three species were in the following order: *I. balsamina >> I. tomentosa >> I. lawii* (Fig.4) Differences in visitation levels were highly pronounced. In Kas, visitation levels were in the order: *I. tomentosa > I. lawii* although the difference was not as pronounced as in Chalkewadi. ANOVA result for the same also supports the fact that there was a significant difference in visitation levels of three species. ($p = 0.0022$)

Table 4: ANOVA table Total individual visitation with plateaus (Kas and Chalkewadi) and species (*I. lawii* and *I. tomentosa*) as factors for alpha=0.05

Sources of variation	**df**	**P value**
Plateau	1	NS
Species	1	**0.0174**
Plateau*Species	1	NS

NS: Not significant

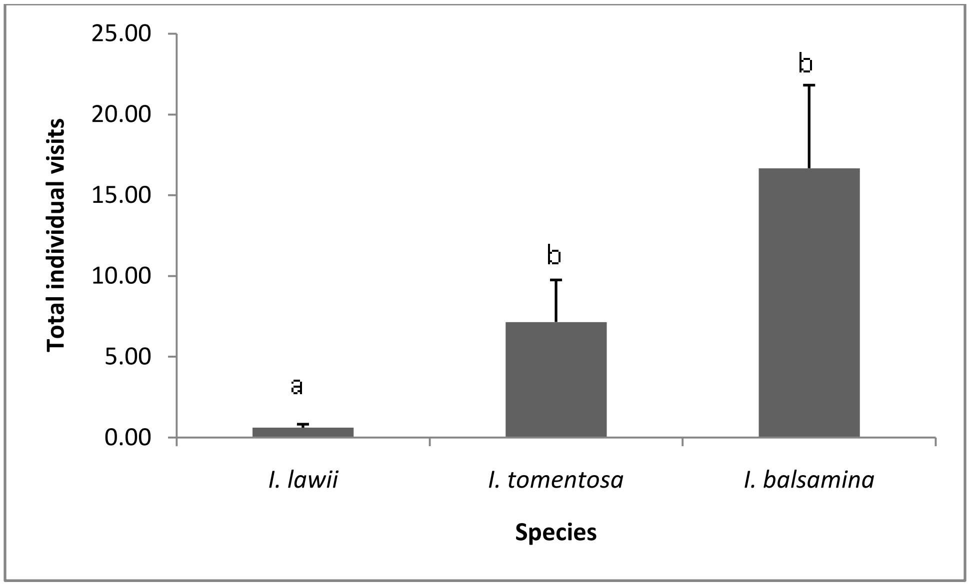

Fig. 4: Comparisons of means of total individual visits for three species (*I. lawii, I. tomentosa, I. balsamina*) on Chalkewadi plateau. Significant means were derived from post-hoc Tukey's HSD test and are indicated by different letters. Error bars indicate 1 std. error of mean

Results for both the parameters chosen to assess visitation were identical for these analyses. Hence results for only Visited number of individuals are presented here.

Result of factorial ANOVA (in a model balanced by excluding *I. balsamina* data) with plateaus and species as factors (Table 4) showed the following: Total visitor number was

Table 5: t-test results for total pollinator visitations with plateaus on Kas and Chalkewadi; alpha=0.05.

Species	**Sources of variation**	**df**	**P value**
I. tomentosa	Plateaus	17	NS
I. lawii	Plateaus	17	**<0.01**

NS: Not significant

greater in Kas than in Chalkewadi but the difference was non-significant. There was a significant difference for species for both plateaus. Comparing the interaction terms indicated by Table 5 and Fig. 5, *I. lawii* showed the great difference in total visits for two populations. Whereas *I. tomentosa* populations showed no significant difference in total visits.

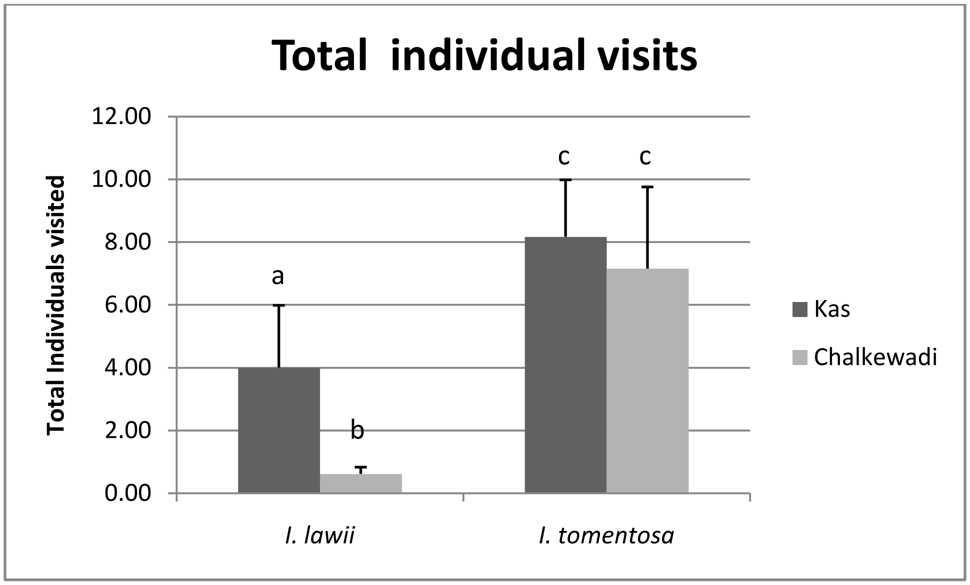

Fig.5: Total individuals visited for two species (*I. lawii and I. tomentosa*) on two plateaus(Kas and Chalkewadi). Significant differences were concluded from t-test (for independent samples) and are denoted by different letters. Error bars indicate 1 std. error of mean

Leaf morphometry: When leaf areas for two species on three plateaus were compared, differences in means of species were apparent. However the differences between three populations of both the species were not significant. (Table 6 and Fig.6)

Table 6: P values of ANOVA on means of Leaf area with plateaus(Kas,Thoseghar and Chalkewadi) and species(*I. lawii* and *I. tomentosa*) as factors. alpha=0.01

Sources of variation	dF	**P values**
Plateau	2	NS
Species	1	**<0.0001**
Plateau*Species	2	NS

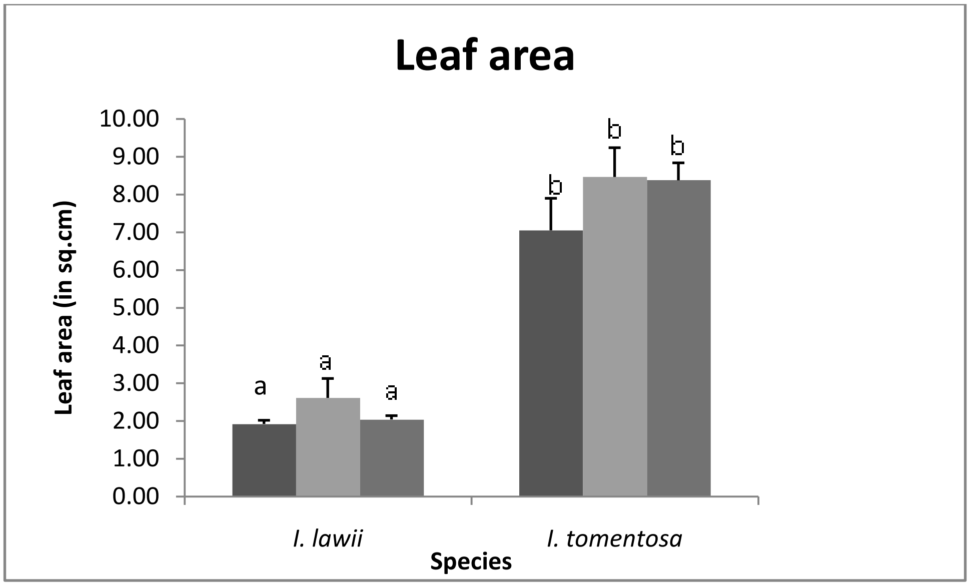

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Fig 6: Means of Leaf area parameter for two species(*I. lawii* and *I. tomentosa*) on three plateaus (Chalkewadi, thoseghar and Kas). Significant differences were concluded from post-hoc Tukey's HSD test and are denoted by different letters. Error bars indicate 1 std. error of mean

Other vegetative traits: From table 7 and Figure 7, All the traits were significantly different for most of the species and plateaus. Comparison of species showed very strong differences for two traits (F=186 for Height and F=88 for leaves/branch, df=2) otherwise the differences in means were very weak (F values ranging from 2 to 7, df=2). Within species, the populations didn't show any significant differences for any trait. Most of the interaction term was driven by weak trends in *I. dalzellii* populations.

Table 7: P values of factorial ANOVAs performed on three vegetative traits: Height, number of leaves, number of lateral branches using plateaus (Chalkewadi, Thoseghar and Kas) and species (*I. lawii*, *I. tomentosa* and *I. dalzellii*) as factors. alpha=0.05

		Height	**Number of leaves**	**Branches**
Sources of variation	dF	**P value**	**P value**	**P values**
		0.0005	**0.0304**	**0.0076**
Species	2	**<0.0001**	**<0.0001**	NS
Plateaus*Species	4	**0.0025**	**0.0054**	**0.0002**

NS: Not significant

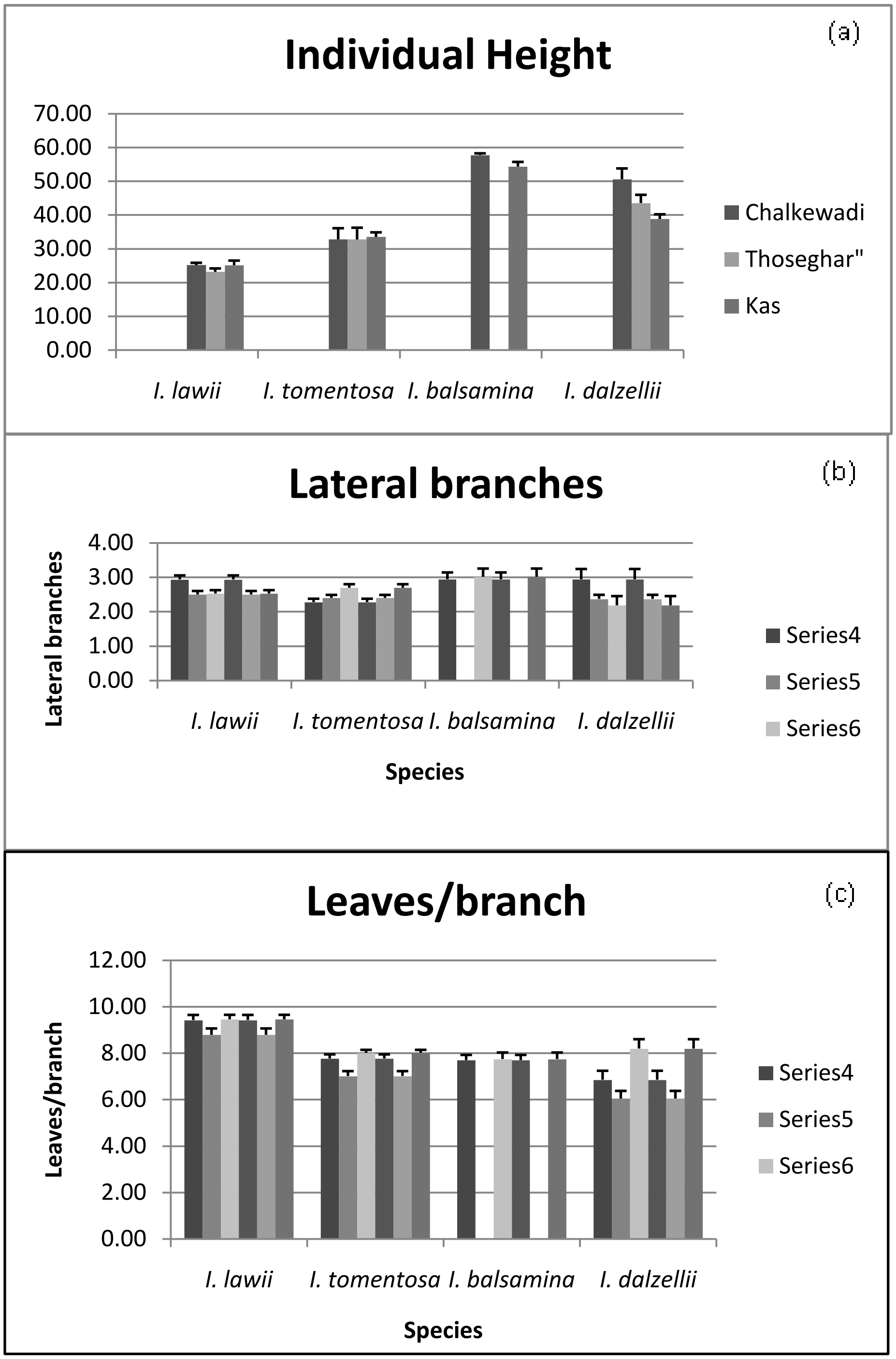


Figure 7: Means of (a)Individual Height, (b) Number of leaves/branch and (c) Number of lateral branches for four species (*I. lawii*, *I. tomentosa*, *I. balsamina*, *I. dalzellii*) for three plateaus(Chalkewadi, Thoseghar and Kas). Error bars indicate 1 std. error of mean

Flower morphometry: Chalkewadi population showed dramatic difference in the flower color distribution where flowers with pink shades were almost absent on the plateau. On Kas and Thoseghar, flower color was predominantly in the pink shades but the lavender colored flowers occurred in varying degrees of frequency. (Fig. 8)

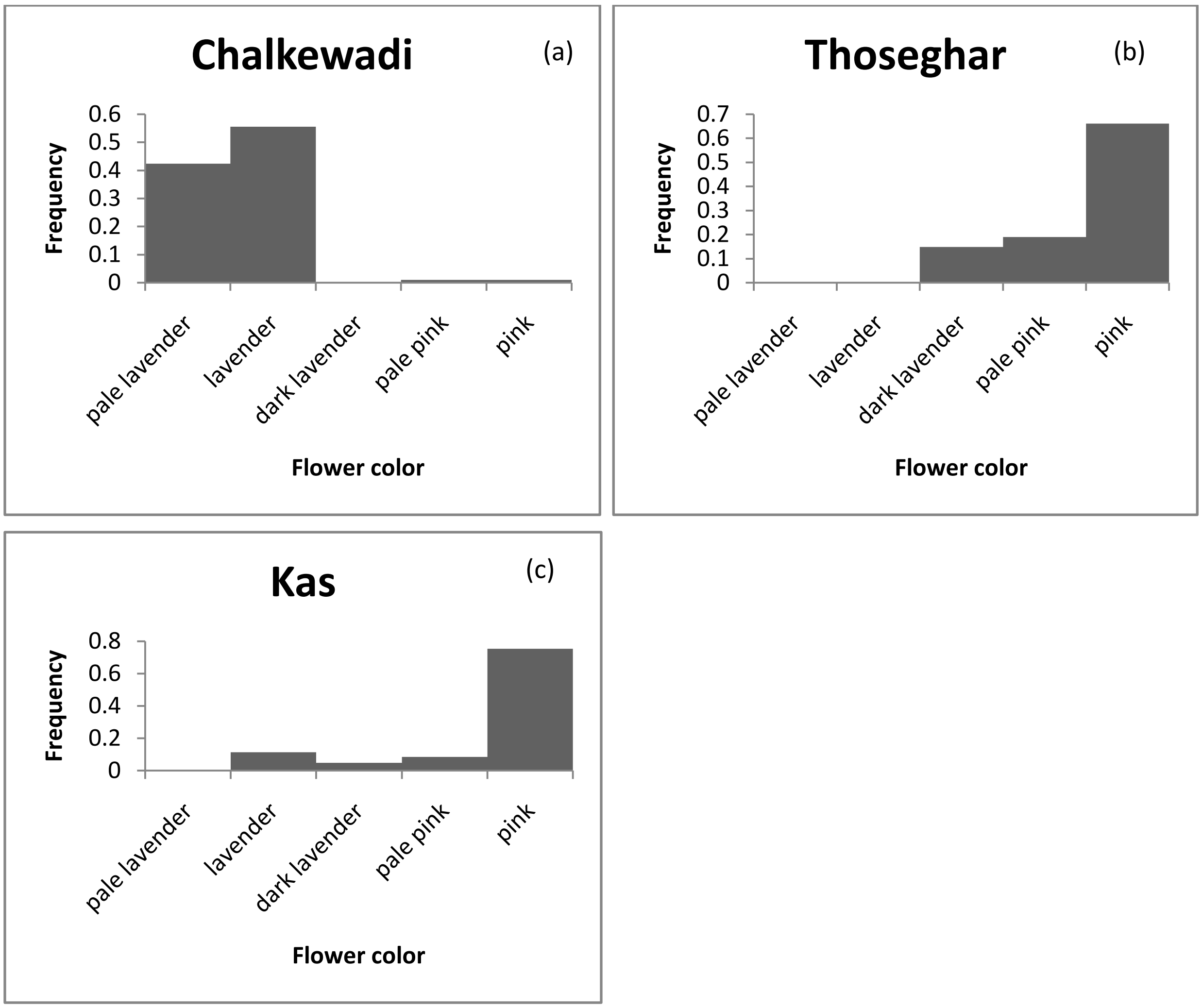


Fig.8: Flower color distribution of *I. lawii* on (a) Chalkewadi, (b) Thoseghar, (c) Kas.

Flower morphology traits: Following results appeared when ANOVAs for each *Impatiens* species were performed using plateaus as factors: (table 8 and fig. 9)

I. dalzellii and *I. lawii* populations showed significant differences in most of the flower traits while *I. tomentosa* populations didn't show differences in 6 out of 9 traits.

For the population of *I. lawii* on Chalkewadi, all the parameters for this population differed significantly from other *I. lawii* populations and by good margin. (Fig. 9)

Table 8: P values of univariate ANOVAs for all flower morphometry traits with only plateaus (Chalkewadi, Thoseghar and Kas) as factors.

	I. dalzellii	***I. tomentosa***	***I. lawii***
Traits	***P value***	***P value***	***P value***
Pedicel	**<0.01**	**<0.01**	**<0.01**
Flower length	**<0.01**	**<0.01**	**<0.01**
Standard petal	**<0.01**	NS	**<0.01**
Corolla opening width	**<0.01**	NS	**<0.01**
Corolla opening height	**<0.01**	NS	**<0.01**
Anther- base	**<0.01**	NS	**<0.01**
Stigma width	**<0.01**	NS	**<0.01**
Anther Height	**<0.01**	NS	**<0.01**
Style height	**<0.01**	**<0.01**	**<0.01**

NS: Not significant

Results of comparing other reproductive traits over the populations of each *Impatiens* species did not reveal any significant gradual trend across plateaus. Although *I. lawii, I. dalzellii* and *I. tomentosa* showed much more differences in these traits than *I. balsamina*.(table 9) Although the differences between the populations of these species were significant, the differences in their mean values were very low when compared to the flower morphometry traits. (Fig. 10 and Fig. 11)

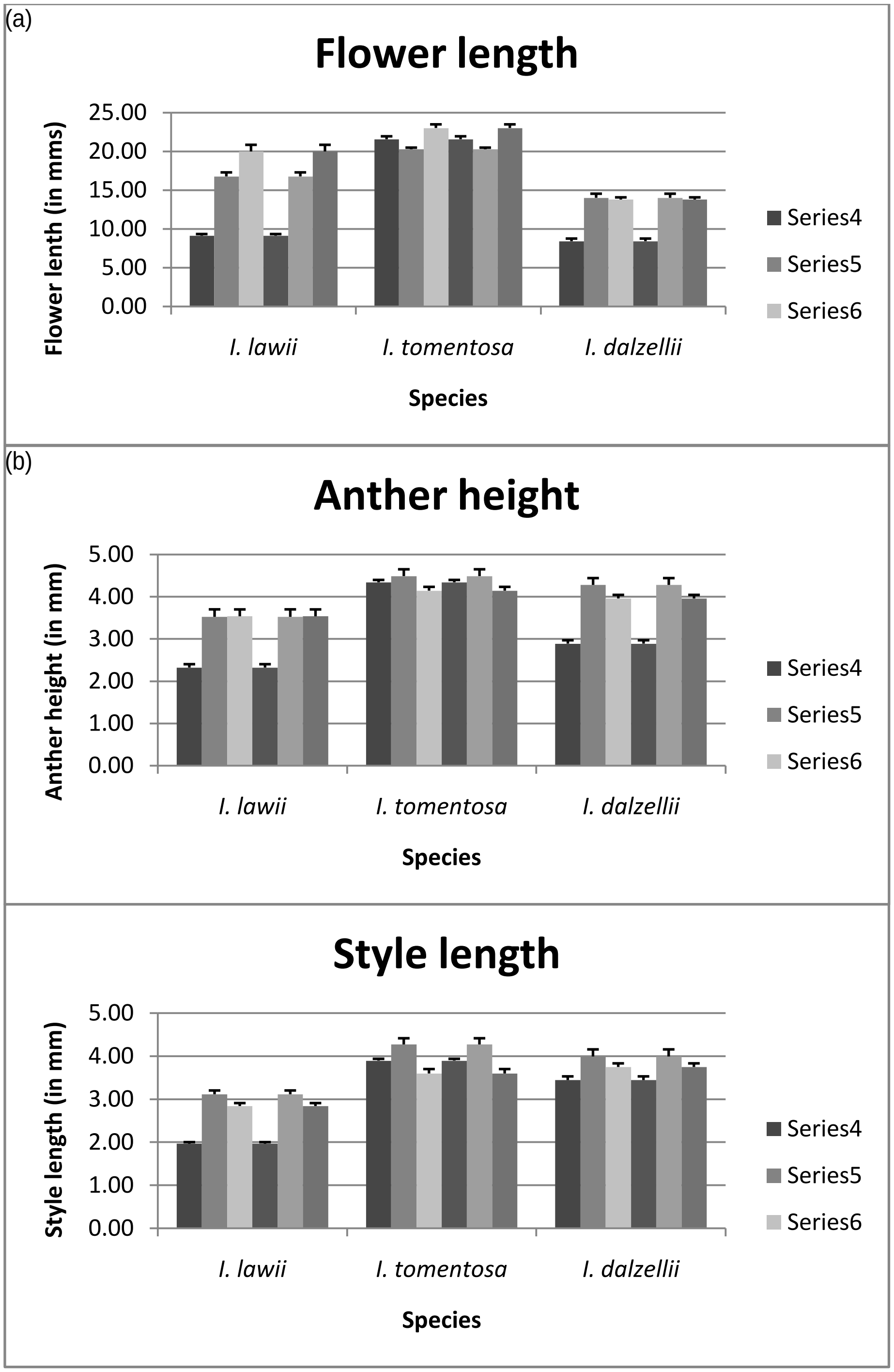


Fig. 9: Flower morphological traits: (a) Flower length (b) Anther height (c) Stigma height for three species (*I. lawii, I. tomentosa and I. dalzellii*) and for three plateaus (Chalkewadi, Thoseghar and Kas). Significant differences are concluded from post-hoc Tukey's HSD test with alpha = 0.05. Error bars indicate 1 std. error of mean

Table 9: P values of univariate ANOVAs performed on reproductive traits for four species (*I. lawii, I. tomentosa, I. balsamina and I. dalzellii*) with plateaus (Chalkewadi and Kas for *I. balsamina*, Chalkewadi, Thoseghar and Kas for others) as factors. df indicates degrees of freedom for the source parameter. Alpha=0.05

Traits	***I. balsamina***	***I. dalzellii***	***I. lawii***	***I. tomentosa***
Df	***1***	***2***	***2***	***2***
	P value	***P value***	***P value***	***P value***
Flowers/branch	**0.002**	NS	**0.0007**	**0.0006**
Fruits/branch	NS	NS	**< 0.0001**	NS
Seeds/fruit	NS	**0.0353**	**< 0.0001**	**< 0.0001**
Seeds/individual	NS	**<0.0001**	NS	**0.0312**
Fruits/flower	**0.0214**	**0.0337**	**0.0015**	**0.0033**

NS: Not significant

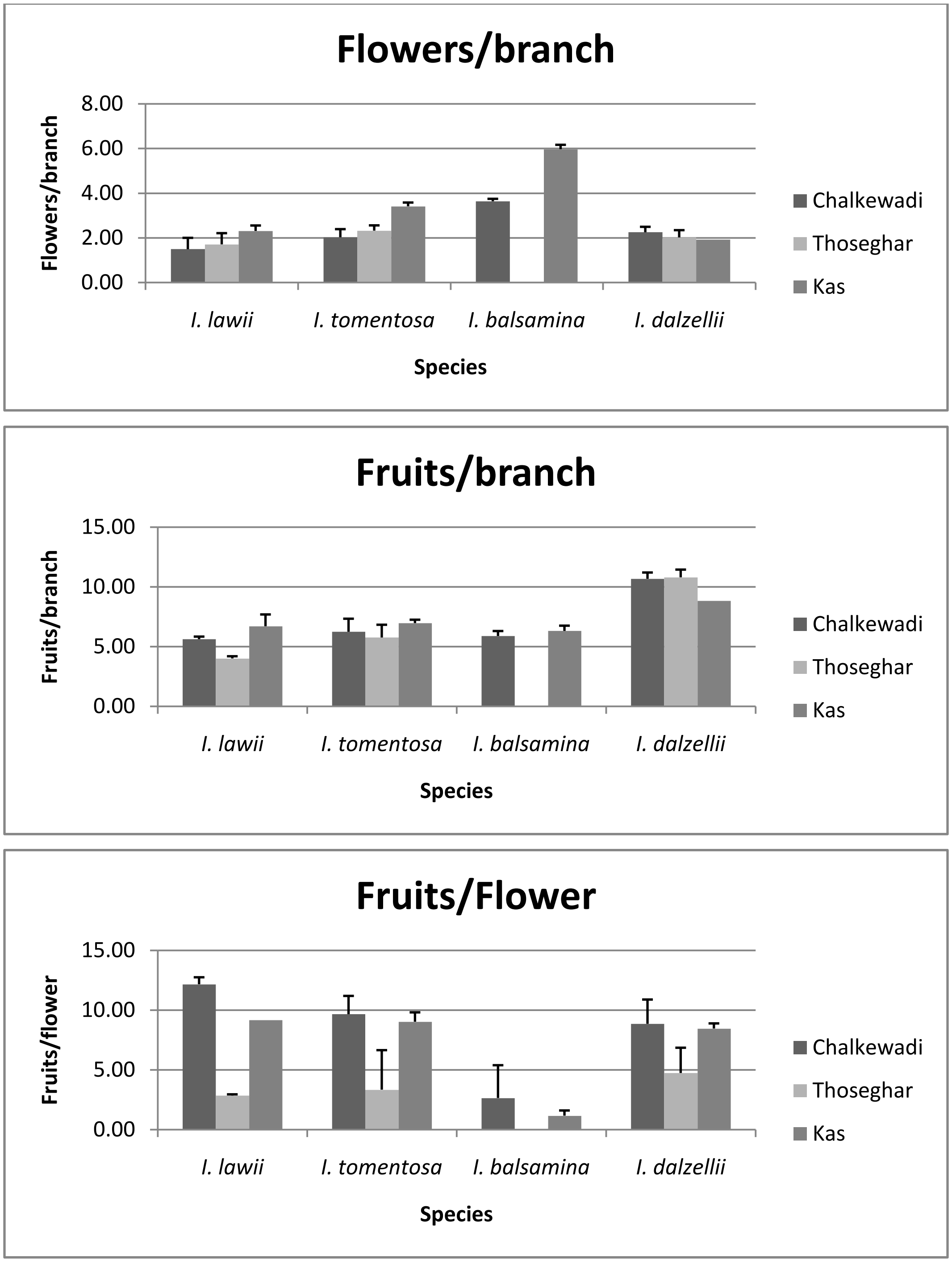
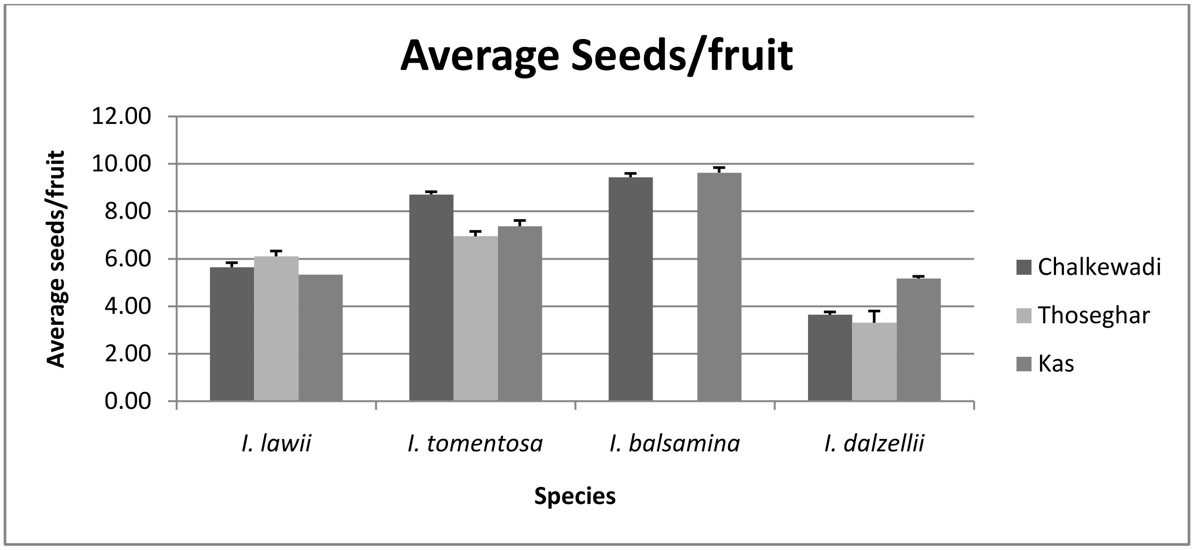


Fig. 10: Means of reproductive trait values: (a) flowers/branch (b) Fruits/branch (c) Fruits/flower for four species (*I. lawii*, *I. tomentosa*, *I. balsamina* and *I. dalzellii*) on three plateaus (Chalkewadi, Thoseghar and Kas)(For *I. balsamina*, Chalkewadi and Kas). Significant differences were concluded from post-hoc Tukey's HSD test.



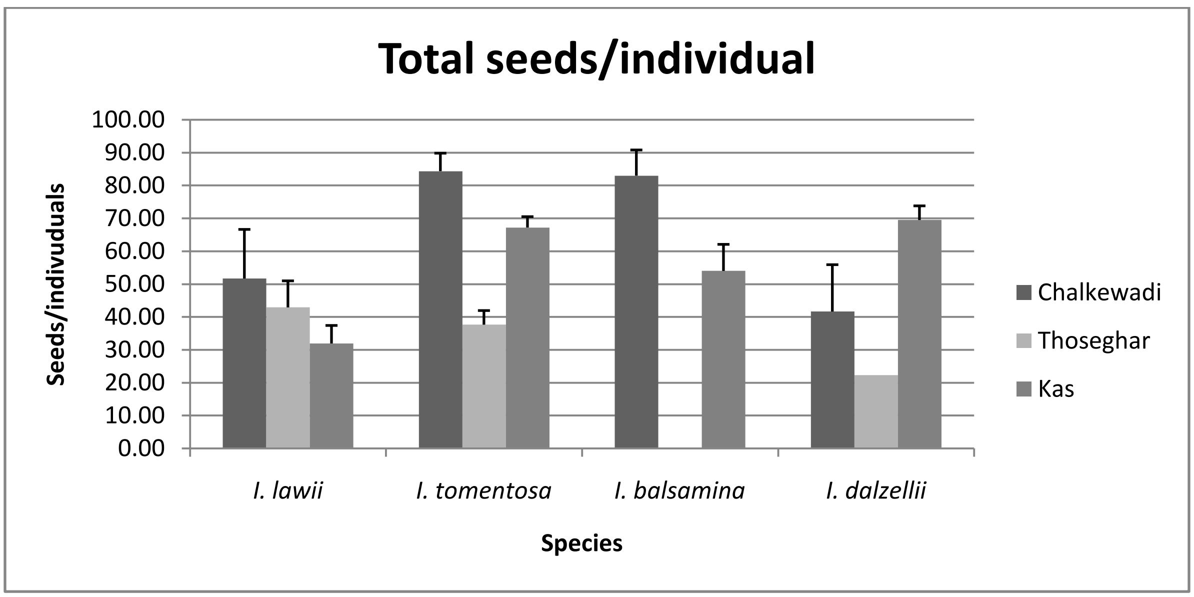


Fig. 11: Means of reproductive trait values: (a) average seeds/fruit (b) total seeds/individual for four species (*I. lawii*, *I. tomentosa*, *I. balsamina* and *I. dalzellii*) on three plateaus (Chalkewadi, Thoseghar and Kas)(For *I. balsamina*, Chalkewadi and Kas). Significant differences were concluded from post-hoc Tukey's HSD test.

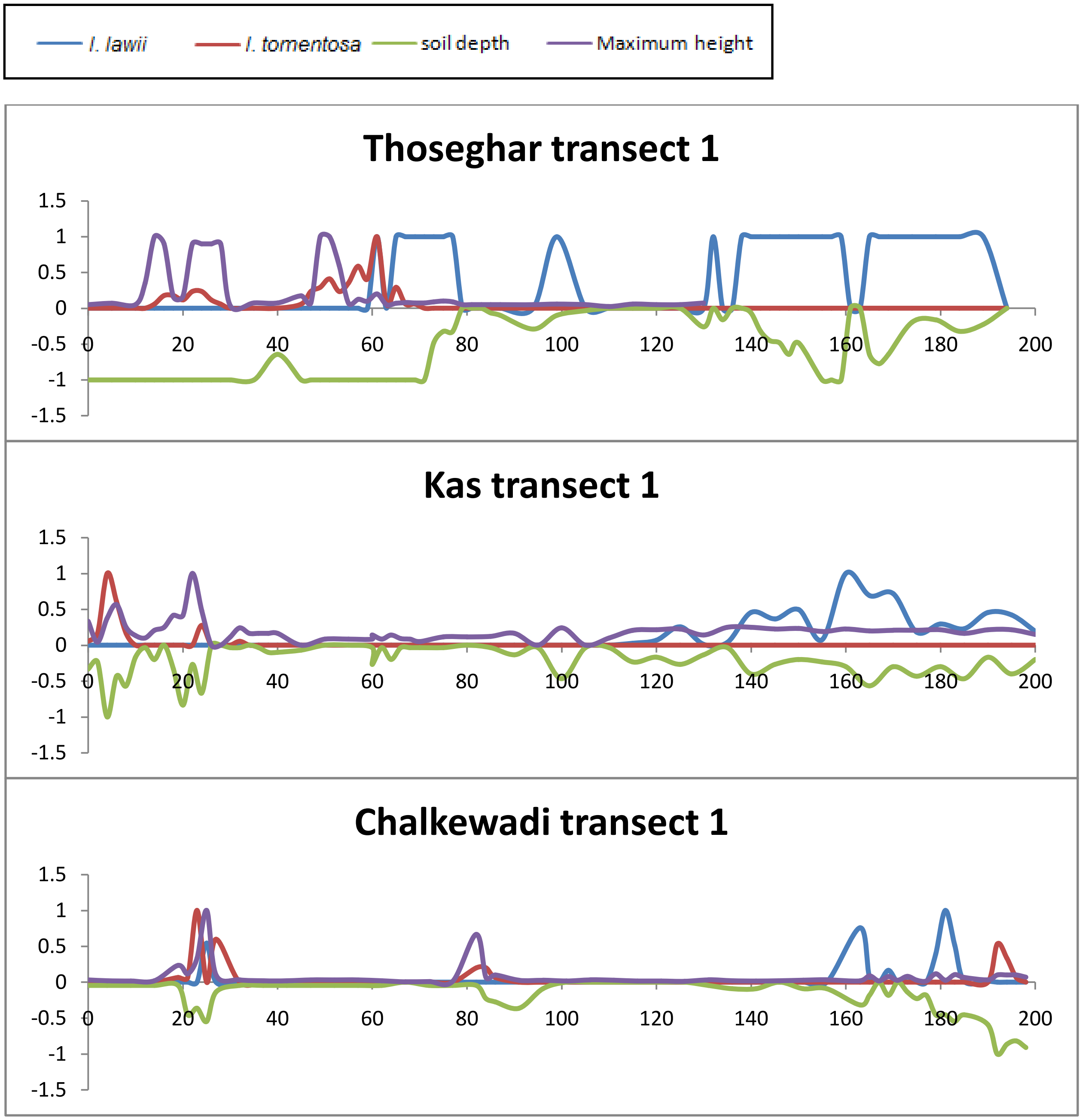


Fig. 12: Graphical representation of the habitat and species presences in three sample transects. In these diagrams, four parameters, *I. lawii* abundance, *I. tomentosa* abundance, Soil depth and Maximum vegetation height are used in order to provide an idea about the general distribution of *Impatiens* species along the microhabitats such as shrubby areas, grassland, streams which have characteristic soil depth and maximum height ranges.

Line Transects:

From table 10 and 11, It was observed that the neighboring habitat parameters better represented the species parameters that the plots habitat parameters. Some key observations were as follows: Presence of *I. tomentosa* was found to be correlated with shrubby areas while for *I. lawii*, presence of water bodies' most significantly related microhabitat. Grasslands or low soil depth could be correlated with the presence of water body but the lack of correlation with both of these parameters show that water presence is needed for the existence of *I. lawii*.

Table 10: R values of multiple regressions performed on Species abundance parameters using two sets of habitats (Last two columns), habitats in the neighborhood of the plot (Grassland, Shrubs, Karvi, Water body, Rocky patches) and habitats within the plot (Grassland, Water body, Karvi, Shrubs, Rocky patches). Results for first abundance parameter- total number of individuals in the plot. Significant R values are highlighted (alpha=0.05) and for significant values, habitat showing significant correlation are noted with the nature of relation. (-negative, + positive)

(a) Plateaus	**Species abundance (# individuals)**	**R (neighbor habitats)**	**R (for quadrant)**
Chalkewadi	*I. lawii*	**0.48 (-rocky patches)**	NS
	I. tomentosa	NS	**0.41 (- water, -rock patches)**
Kas	*I. lawii*	**0.5 (-rock patches)**	NS
	I. tomentosa	NS	NS
Thoseghar	*I. lawii*	**0.40 (+water, +grassland, -shrubs, -rock patches)**	NS
	I. tomentosa	**0.37 (+shrubs)**	NS

Table 11: Summary of multiple regressions performed on Species abundance parameters using two sets of habitats (Last two columns), habitats in the neighborhood of the plot (Grassland, Shrubs, Karvi, Water body, Rocky patches) and habitats within the plot (Grassland, Water body, Karvi, Shrubs, Rocky patches). Results for second abundance parameter- Presence/absence of species in the plot).Significant R values are highlighted (alpha=0.05) and for significant values, habitat showing significant correlation are noted with the nature of relation. (-negative, + positive)

Plateaus	**Species abundance parameters (Presence/absence of individuals)**	**R values of neighbor habitats**	**R values for plot habitats**
Chalkewadi	*I. lawii*	**0.58 (+grasslands, -rock patches)**	**0.28 (+vegetation)**
	I. tomentosa	**0.38 (+shrubs)**	**0.33 (-rock patches)**
Kas	*I. lawii*	**0.67 (- rock patches, -shrubs)**	**0.21 (+vegetation)**
	I. tomentosa	NS	**0.25 (+ shrubs)**
Thoseghar	*I. lawii*	**0.50 (+water, -shrubs, - rock patches)**	NS
	I. tomentosa	**0.63 (-water, -rock patches, +shrubs)**	**0.34 (-bare soil)**

Population estimate: All the estimated population sizes are estimated in table 12.

Table12: Estimates for abundances of *I. lawii* and *I. tomentosa* for 3 plateaus (Kas, Thoseghar and Chalkewadi)

Plateaus	***I. lawii*** **abundance**		***I. tomentosa*** **abundance**	
	number	density**	number	density**
Kas	13.2×10^6	4.4	6.28×10^5	0.209
Thoseghar	2.06×10^6	0.68	3.22×10^5	0.107
Chalkewadi	3×10^6	0.3	11.8×10^5	0.118

*Unit: # individuals ** Unit: # individuals/sq.m. area

Discussion

The five *Impatiens* species examined had distinct spatial distribution patterns. Lawii was found exclusively on the plateaus. *I. tomentosa* was found in the plateaus, and to some extent on the slopes. While both *I. tomentosa* and *I. lawii* were found mostly on the plateaus, their occurrences were mutually exclusive. *I. dalzellii* were present only on the slopes of the plateaus. *I. oppositifolia* were rare and were found in habitats similar to *I. tomentosa*. *I. balsamina* were the most widely distributed and found growing in large clusters with high densities near human habitats, often by the road side. They were rarely found on the plateaus, except on Chalkewadi where there were found along the road and man-made ditches on the plateau. *I. balsamina* were very abundant off the plateaus, in the valleys, and their populations were less fragmented and more continuous compared to other *Impatiens* species.

There were no specific differences in peak timings between species. All the species had flowering peaks between the 8th September to 28th of September. Within species, one notable difference was with the Chalkewadi *I. lawii* population which had an earlier peak on the 8th September while other *I. lawii* populations peaked two weeks later. In Kas, *I. tomentosa* population had a later peak (28th September) while the other plateau populations peaked a week earlier. It is possible that this may result in reduced gene flow between the early or late peaking populations.

There was no significant difference in peak flowering durations for any of the species. Peak flowering durations ranged from three to six weeks. *I. balsamina* flowered for longer periods, 6 weeks, while *I. dalzellii* populations showed shorter peak flowering periods. In general, flowering peak durations of all the populations for each species had enough overlap between them to allow significant gene flow between them.

The major flower visitor species observed for these *Impatiens* species in these plateaus are: *Apis dorsata, Apis florea*, other unidentified bee species, a group of fly species, a group of moth and butterfly species, a group of beetle species and ants. There was not much difference in the diversity of visiting species the plateaus and species. The one exception was for *A. dorsata* which was not observed to visit *I. lawii* flowers on either of plateau. Another striking difference was that only three of the nine species visited *I. lawii* on Chalkewadi whereas Kas *I. lawii* flowers were visited by eight of the nine species.

Comparing the number and time spent by visitors in Chalkewadi, it was very evident that *I. balsamina* had a greater number of visitors than *I. tomentosa* and *I. lawii*, while *I. lawii* had exceptionally low number of visitors. Comparing *I. lawii* and *I. tomentosa* between Kas and Chalkewadi, showed no difference in total visitations between the two plateaus. However, the number of individuals visited to *I. lawii* in Chalkewadi, was significantly lower than the number of individuals visiting this species in Kas. The difference in visiting species could play a vital role in determining the maximum range at which a gene flow can occur. For example, out of all visiting bee species, *A. dorsata* is the biggest species and has a maximum foraging range which extends up to 10 kms. (Dyer and Seeley, 1991) (Greenleaf et. al. 2007) Absence of visitation from these species could be a crucial factor explaining variations in plateaus that are so close to each other.

To understand the differences between visitations, one needs to take into account floral traits as well as floral display sizes. *I. balsamina* which had greater visitation also had a greater number of flowers per individual. *I. balsamina* also had the biggest flowers among three species while *I. lawii* had smallest flowers. Although the pollinator preference could be affected by rewards per visit about which we don’t have any information for our set of *Impatiens* species. Flower color and flower size can also explain the difference in two *I. lawii* populations. There was a significant shift in flower color for the two populations where on Kas most of the flowers had dark pink shades and on Chalkewadi almost all the flowers had lighter bluish lavender shades. Also, *I. lawii* flowers on Chalkewadi were almost half the size of flowers on Kas. These factors could have made significant changes in flower visit preferences. Low levels of flower visitation on Chalkewadi *I. lawii* flowers may also result from competition for attracting visitors by *I. balsamina*, which are absent on Kas.

On Thoseghar, *I. lawii* clusters exist having different sets of flower color. Comparative analysis of pollinator visitation can be performed to see if the flower colors change the preferences of pollinator species. Experiments with controlled pollination levels (preventing pollination, hand pollination, species-mediated pollination) can performed and the reproductive success in form of fruits/flower or seed number and seed viability can be assessed to study the relation of flower color can be done. Through this experiment added with cross-pollination experiments, modes of breeding (which generally are conserved for species) can also be assessed. . In this study, nocturnal pollinator visitation was not studied and could be the major contributors in total pollinator visitation. Also, post-zygotic reproductive processes such as dispersal and germination success should also be studied.

Observing the dramatic flower color and size differences, following could be the cases 1. that the populations of *I. lawii* on Kas and Chalkewadi could be genetically distinct from each other or 2. These could be two variants of the species or at least 3. Chalkewadi populations could be the diverging population.

To confirm these possibilities, it should be confirmed that the flowering traits showing a difference are indeed genetic traits. As a start, translocation of individuals could provide an initial idea about this hypothesis. Also, reproductive compatibility between these populations could also be checked by crossing individuals from these two populations.

Vegetative traits are highly plastic, not species-specific and depend on the environmental conditions experienced by the plants. Traits like height, number of leaves and number of lateral branches are expected to be dependent on the light environment. This in turn will depend on the density of plants, height of neighbors in the immediate environment of the plant. Plants growing in more open less dense environments like that for the Chalkewadi. *I. lawii* will show shorter height, and more lateral branches, while the higher density of conspecifics and lower light availability in Kas would result in taller plants with less lateral branching. This might explain the high variation seen in vegetative traits in these species. However for leaf area, which is a species specific trait, there were differences between species, but not within species, between populations from the different plateaus, as expected.

Reproductive traits, like flower morphology are highly conserved and species specific. Surprisingly, there were significant differences in floral morphology for *I. lawii* and *I. dalzellii* species especially for Chalkewadi and Kas populations. For *I. dalzellii*, the differences could have emerged from low populations sizes and their high habitat specificities which could restrict the gene flow and promote the divergence between populations.

However, other reproductive traits derived from phenology data like the number of fruits had high variation, as may be expected. Further experiments which include the monitoring of individuals is important to conclusively comment on the variations between them.

Comparing all *Impatiens* species, *I. lawii* and *I. tomentosa* seem to coexist on rocky plateaus. But when plateau space was divided in further classes of habitats i.e. microhabitats, very significant trends both for *I. lawii* and *I. tomentosa* were observed. For all the plateaus, *I. tomentosa* seemed very closely related to Shrubby areas. Now, the natural requirements for the existence of *I. tomentosa* could either be deep soil or the shade which are linked with the shrubby areas. Other factors could be active/ passive species interactions. E.g. It was observed that Neanotis spp. individuals were almost exclusive of the presence of *I. tomentosa*. Species generally occurred on the boundaries of shrubby areas. Also, deep soil could also be the requirement for these species.

Similarly, for *I. lawii*, presence of water bodies in the vicinity was the most significantly related microhabitat. Grasslands or low soil depth could be correlated with the presence of water body but the lack of correlation with both of these parameters show that water presence is needed for the existence of *I. lawii*. Other idea of restriction of *I. lawii* in the vicinity of water could be the active competition from other species. *I. tomentosa* or other associated species could themselves be the candidates for competition. Therefore, *I. lawii* and *I. tomentosa* share the same rocky outcrops but seem partition themselves to the specific microhabitats. The occurrence of *I. Lawii* and *I. tomentosa* were mutually exclusive, and the negative correlation between presences of *I. lawii* and *I. tomentosa* supports this conclusion.

Population parameters, though roughly estimated show the trend that Kas populations were larger and dense than other two plateaus.

Species occurring on rock outcrops are generally highly specialized in their physiology and general biology (Watve, 2007) to efficiently interact with the surrounding environment and the community*. I. lawii* and *I. tomentosa* show the example of partitioning of microhabitats to sustain together. Such specialist species show high endemism levels and most of these have endangered status. (Watve,2007)

Introduction of any new species which are generalist in nature could disrupt the interactions within the community and outcompete some species. *I. lawii* is a critically endangered species (Watve, 2007) and it seems that it is being restricted by the shrubby areas. Karvi, generally a part of the shrubby areas are generalists in nature and can grow even in shallower soil areas. On Kas and Chalkewadi, Karvi seems to restrict the spread of *I. lawii* on grasslands regions. So, spread of such damaging species should be prevented in order to prevent the reduction in *I. lawii* populations. Tourist visits might pose a major problem for the survival of such endangered species. Large scale conservation efforts are needed to prevent the extinction of these species. Similarly, I. dalzellii, which occur on the slopes and require presence of shrubs to persist, are an endangered species. Very rare and low density populations of I. dalzellii were found on the slopes of the plateaus. Rapid deforestations, grazing animals, and growing prevalence of terrace farmlands could become the reason for local extinctions of these species.

Further work:

Many factors related to the ecology and reproductive biology of the *Impatiens* species were not studied in this study. Extensive analysis of biogeography and establishment history of these species should be performed to gather the temporal and geographical distribution of these species. Information about this can be gathered from various herbaria. Use of genetic markers to assess the genetic variation can strongly conclude about the gene flow between the populations of the species. Pilot study has been performed for designing the experiments to do the population genetics study.

Most of the characteristics of selected species are similar except for the population parameters and geographic parameters. Hence, these parameters could play a major role in deciding variation within and between species.

It seems that looking from the correlations provided in table 1, endemism, population structures, pollinators could be major ecological determinants causing the differences in inter-population variations.

I. lawii populations on Kas and Chalkewadi showed some crucial differences for reproductive traits. Significances in these differences reflect on their general ecology. e.g. changes in flower color and flower size seem to have affected the flower visits for these populations. Although, there was no entire shift of pollinator assemblage, larger species seemed to avoid the *I. lawii* individuals on Chalkewadi. Further dynamics could lead to gradual shift of pollinator assemblage and may lead to rapid divergence of population. (Schemske 1978)

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Appendix:

Genetic analysis:

A pilot study was performed to set up an experiment of genetic analysis using different types of markers.

Summary of molecular markers analysis:

Very general idea behind detecting genetic variation is to study variation at one or many loci in genomic/organelle DNA or a sequence variation in conserved proteins. The sources of variation are most commonly the neutral mutations which result in changes in sequences or deletion or the duplication of stretches of DNAs. In such cases, the variation occurs in the form of changes in sequences or the changes in number of repeats of some stretches of DNA. This variation can be detected by differentiating isolated DNAs in the forms of sequence, mass or size. Most common and practical techniques to separate the DNA based on their properties are gel electrophoresis and western blot.

Rates of changes of these variations can also be detected if the information about the mutational mechanisms is available for the specific type of sequences or loci. Based on this information and other practical aspects, large number of genetic markers have been developed and used extensively. Appropriate statistical tools to analyze the data have also been developed.

The success of finding a reasonable amount of variation using a specific kind of marker depends on following factors:

1. Intrinsic properties of markers such as information about the mutational mechanisms.
2. Chances of specific genetic markers working Vary according to the choice of the species.
3. How closely are the samples genetically related to each other? Are these different species or are these the different populations of the same species. Because for the first case, any non-specific marker could work, since chances of

detecting the variation in two distant species is very high compared to the second case.

There are two broad categories of molecular markers: Dominant and Co-dominant.

Dominant markers are those markers which mask the expression of other counterpart on the second copy of the gene in diploids. Thus using these markers does not yield information about the heterozygocity of an individual. Dominant markers generally are non-specific to any loci and can amplify sequences at multiple loci at the same time using PCR. Their usual mechanism is random amplification using pre-determined markers. Reliability of these markers to detect variation in a given species or for multiple species is variable since the amplification is random and there are chances of missing some extent of variation. They are useful for comparing genetically distant samples. Being non-specific, they don't require any information about the sequence to be analyzed. They are more easy and cost-effective to use. Examples of such markers are AFLP, RAPD and other derivations from these methods.

Co-dominant markers are the sequences specific to the loci and amplify the sequences only on the given loci. Their working mechanism of to amplify the conserved part of the loci and resolve the difference in sequence size occurring due to duplication events. Being very specific to the locus, they can exhibit high reliability in predicting the variation. Also for the analyses where within species differences are to be assessed, co-dominant markers are more effective and reliable than the dominant markers. One major drawbacks of using such markers are that the sequence information about the loci is needed which can be time and cost consuming. But if the sequence information about the loci for our choice of species is available on various databases, these markers can be most useful to detect variation. Examples of such markers are RFLPs, microsatellites.

Use of microsatellite marker is recommended for this exercise since the samples to be analyzed are the populations of the same species which discourage the use of the dominant markers.

Summary of microsatellites:

Microsatellites are highly prevalent tandem repeats of 2–6 nucleotides found in the nuclear genomes of most taxa. The no of repeats generally vary from 5 to 40 but more repeat can also exist. Dinucleotide repeats are the most abundant types of microsatellites. Flanking regions of these repeats are well conserved within and sometimes between closely related species. Using these sequences as a template to synthesize the primers, which in turn can amplify the microsatellite region. This method can provide the information about the variability in number of repeats of the short sequences. Microsatellites have high mutation rates (Average 5 x 1o^{-}4) that generate the high level of allelic diversity which allows to study the process of divergence or in general species dynamics on a relatively shorter scale.
These properties allow microsatellites ideal candidates for analyzing within species differences. Only issue with using the microsatellites for this study is that there is no sequence information available about the satellites for our set of Impatiens species. Development of primers to perform the analysis would require longer time and higher cost. However microsatellite studies have been performed on 2 of the impatiens species (*I. glandulifera* and *I. lateristachys*) found in southern china and Himalayan ranges. If one of these species are genetically closer to our set of species, the primers developed for them could be tried for our species. Phylogeny of large number of species of *impatiens* genera, which includes *I. balsamina*. Morphology based cladograms have also been developed for many of the species. Comparing the morphological parameter sets of *I. glandulifera* and *I. lateristachys* with the set of morphological parameter sets of species available for this work, we might get the idea about which of the two species is closer to our species.

Looking at the comparisons , *I. lateristachys* seems to closer to the species of interests than *I. glandulifera.*

Total microsatellite sequences for impatiens available: 21- 8 for I. *glandulifera* (Himalayan) and 13 for I. lateristachys (Chinese). Out of all developed microsatellite sequences, maximum number of alleles separated were 6 and 5 respectively for above two species. Number of populations: (3 species X 2 populations) = 6 with 25 individuals in 5 and 15 in 1.

Procedure proposed:

1. DNA extraction from leaf tissues of individuals → Find the microsatellite primers which work for our species. → Extract and amplify the microsatellite sequences by PCR using selected primers → Analyze the length variation.
2. In Ge paper, it is mentioned that 42 individuals from 3 populations were selected for finding working microsatellite primers, after which they identified 13 loci with maximum 6 alleles. Another result from Ge paper is that amplification of some of loci failed when primers were examined in closely related species.
3. For genetic variation to be conclusively determined, at least 10 loci should be assessed i.e. 10 primers must be chosen for further studies.
4. PCR protocols may need to be improvised.