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REGULAR PAPER – TAXONOMY/PHYLOGENETICS/EVOLUTIONARY BIOLOGY

Sympatric co-existence of two ecotypes of *Impatiens noli-tangere* (Balsaminaceae) with different morphology and flowering phenology

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Abstract

In angiosperms, intraspecific variation of flowering phenology may affect reproductive isolation and, consequently, speciation. This study focused on *Impatiens noli-tangere* (Balsaminaceae), which is distributed over broad latitudinal and altitudinal ranges in Japan. We aimed to reveal the phenotypic mixture of two ecotypes of *I. noli-tangere* with different flowering phenology and morphological traits in a narrow contact zone. Previous studies have shown that *I. noli-tangere* has early- and late-flowering types. The early-flowering type makes buds in June and is distributed at high-elevation sites. The late-flowering type makes buds in July and is distributed at low-elevation sites. In this study, we analyzed the flowering phenology of individuals at an intermediate elevation site where the early- and late-flowering types grow in sympatry (contact zone). We found no individuals showing intermediate flowering phenology at the contact zone, and early- and late-flowering types were clearly distinguishable. We also found that the differences in many other phenotypic traits between the early- and late-flowering types were maintained, including the number of flowers produced (total number of chasmogamous and cleistogamous flowers), leaf morphology (aspect ratio, number of serrations), seed traits (aspect ratio), and flower bud formation positions on the plant. This study showed that these two flowering ecotypes maintain many different traits in sympatry.

Keywords Contact zone · Flowering ecotype · Leaf morphology · Seed morphology · Trait differentiation

Introduction

In angiosperms, flowering phenology is important for plant reproductive success, and its intraspecific variation may lead to reproductive isolation and speciation (Bronstein 1990; Munguía-Rosas et al. 2011; Rathcke 1983). Multiple ecotypes with different flowering phenology have been observed in many plant species (e.g., Nomura et al. 2022; Toji and Itino 2020), and variation in flowering phenology may contribute to achieving or maintaining the early stages of speciation (Taylor and Friesen 2017). Therefore, it is important to reveal the details of flowering phenology to understand the ecological speciation and diversification mechanisms of plants (Taylor and Friesen 2017).

Impatiens noli-tangere L. (Balsaminaceae) is an annual herb that produces both cleistogamous and chasmogamous flowers (Hatcher 2003). Masuda and Yahara (1994) identified several populations (sites) in the Nikko Mountains of Japan, and reported that the *I. noli-tangere* plants of different populations exhibited different flowering phenology and cleistogamous flower rates. Although the variations were correlated mainly with light conditions, at some sites they detected no relationship between flowering phenology and environmental factors. Thus, the factors responsible for the creation and maintenance of this variation in flowering phenology are not fully known. Therefore, sites where ecotypes exhibiting different flowering phenology are distributed in sympatry, or in parapatry at a fine spatial scale, should be investigated to understand the observed variation in the flowering phenology of *I. noli-tangere*.

Tsubasa Toji and Tetsuya Yamamoto contributed equally to this work.

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In our previous study, we observed two ecotypes of *I. noli-tangere* with different flowering phenology in the Misato Mountain region of Nagano Prefecture, Japan (Shinohara et al. 2015). In this region, an early-flowering type that makes buds from early June and has few chasmogamous flowers is distributed at higher elevations (about 1180–1300 m a.s.l), and a late-flowering type that makes buds from late July or early August and has a large number of flowers is distributed at lower elevations (about 850–970 m a.s.l). Furthermore, individuals with different bud formation seasons coexist at an intermediate-altitude site (contact zone, 1130 m a.s.l.). Therefore, the Misato region, where ecotypes showing different flowering phenology coexist, is an area suitable for studying the creation and maintenance mechanisms of variation in the flowering phenology of *I. noli-tangere*. So far, however, the flowering phenology at the contact zone of early-flowering and late-flowering types has not yet been reported at the individual level. As a result, it is not known whether the two types with distinctly different flowering phenology are distributed sympatrically at the contact zone or whether intermediate types are present.

In this study, we focused on the Misato Mountain region where the two types of *I. noli-tangere* coexist and analyzed the flowering phenology of early- and late- flowering types at the individual-level. In addition, to clarify trait differentiation between the two types, we measured leaf traits, seed traits, number of flowers produced, and the position of first flower bud formation. Using these data, we investigated whether dimorphism was maintained in the flowering phenology and other traits of *I. noli-tangere* at the contact zone, and whether intermediate individuals were present.

Materials and methods

Plant species

Impatiens noli-tangere (Balsaminaceae) is an annual herb distributed in the cold temperate zone of the Northern Hemisphere, and in Japan it is found throughout the country except in the Nansei Islands (Hatcher 2003; Satake et al. 1982). This species produces both cleistogamous flowers, which reproduce by self-pollination, and chasmogamous flowers, which have funnel-shaped lower sepals and can be cross-pollinated by bumblebees (Hatcher 2003). Chasmogamous flowers are resupinate with a 180° twist, and each flower has three sepals and five petals. The flowers are yellow overall with small red and brownish spots. Flowers with different reproductive modes can be identified by whether the buds open before forming fruit. Cleistogamous flowers form fruit in the buds without opening, whereas chasmogamous flower buds open before forming fruit.

This study was conducted in the Misato Mountain region, Azumino, Nagano, Japan (137.831082 E, 36.281420 N), where two ecotypes of *I. noli-tangere* were recorded (Shinohara et al. 2015). In the same way as the Misato Mountain region, a marked variation in flowering phenology is observed in the Nikko Mountain region, Japan, but this variation is relatively gradient (Masuda and Yahara 1994).

Distribution of the two ecotypes

To determine the detailed elevational distribution of the two types of *I. noli-tangere* in this region, we conducted a distribution survey of *I. noli-tangere* at elevations of 800–1400 m during the flowering seasons (June to October) of 3 years, 2013, 2015, and 2021. Because various plant traits differ significantly between the ecotypes (as mentioned in the Results), we could readily distinguish between the early- and late-flowering types.

Furthermore, we identified the distribution patterns of the two ecotypes in the contact zone by observing their distributions at the individual level at three sites at elevations of 1045, 1118, and 1130 m.

Flowering phenology and flower reproduction

We observed the flowering phenology of *I. noli-tangere* approximately weekly from May to October 2013 at an elevation of 1130 m in the Misato region, where both early-flowering and late-flowering types were distributed. Each *I. noli-tangere* individual was labeled so that the same individuals could be identified at each observation time. Of the 17 labeled individuals that survived to the flowering stage, the 10 individuals with buds on June 23, when cleistogamous flowers were first observed in the population, were considered to be the early-flowering type. We used this date as the base date (day 1) and calculated subsequent dates as the number of days elapsed from that date. The remaining seven individuals began to form buds on July 23, and we considered those to be the late-flowering type. Thus, we hereafter define individuals with buds by June 23 as the early-flowering ecotype and those that began to bud on July 24 as the late-flowering ecotype. The differences in leaf morphology, which we discuss later, confirmed that distinguishing between the early-flowering and late-flowering types in this manner was valid. The survey of the 10 individuals identified as early flowering was terminated on September 11, 2013, because all 10 individuals had died by then. The remaining seven individuals were surveyed until October 4, 2013. To compare the flowering phenology between the two types, the starting date of flowering was compared by Mann–Whitney U-test. In addition, the date on which the greatest number of chasmogamous flowers was blooming during the flowering period of each individual was treated as

its peak flowering date, and the peak flowering date between the two ecotypes was compared by Mann–Whitney U-test. All statistical analyses in this study were performed with R Software ver. 4.1.2 (R Core Team 2013).

All buds were labeled on each individual plant, and, at a later date, whether the buds were chasmogamous or cleistogamous flowers was recorded. For the determination of the timing of flower production, chasmogamous flowers were treated as produced when the buds formed petals and opened, whereas cleistogamous flowers were treated as produced when the buds formed fruit without having opened. Student's t-test was used to compare the total number of flowers produced by each individual between the two types. Mann–Whitney U-test was used to compare the proportion (%) of chasmogamous flowers relative to the total number of flowers produced by each individual between the two types.

Leaf morphology

To quantify leaf shape differences between early- and late-flowering types, we measured the leaf aspect ratio and the number of leaf serrations. First, we collected three leaves per individual from 15 to 21 individuals of each population at four sites in the Misato region in 2013 (two sites were occupied by only early-flowering type individuals, and the other two sites were occupied by only late-flowering type individuals). We collected mature leaves with petioles longer than 1 cm and without any insect damage from the upper half of the plant during the flowering period of *I. noli-tangere*. We brought the sampled leaves back to the laboratory and pressed them (Fig. 1d). The leaf specimens were digitally imaged using a scanner (CanoScan 9900F, Canon), and the leaf length (LL) and leaf width (LW) were measured with Photo Measure image analysis software (Kenith Corporation). The leaf shape index (LL/LW) was then calculated from the measured leaf length and leaf width. The number of leaf serrations was also counted. If the number of serrations could be counted on only one side of the leaf, the number of serrations on the entire leaf was estimated under the assumption that the leaf was linearly symmetrical. The calculated leaf shape index and the number of serrations of the leaves from each individual plant were averaged and used as the representative value for that individual. Comparisons among populations were made using the Mann–Whitney U-test.

Discriminant analysis was also conducted using leaf length, leaf width, and number of serrations as covariates to test whether leaf morphology could be used to discriminate between the early- and late-flowering types.

Seed morphology

To compare seed morphology between early- and late-flowering types, 1–5 seeds from each of the 14–16 individuals of each type were collected at the contact zone at 1130 m elevation in the Misato region in 2013. Seeds of the early-flowering type were collected in early July 2013, and seeds of the late-flowering type were collected in September 2013. Seeds from the cleistogamous and chasmogamous flowers were measured separately. The collected seeds were photographed on paper with a 1 mm^2 grid under a microscope (Fig. 1e). Then, the seed length/width ratio was measured using ImageJ software (Schneider et al. 2012). The average of the measurements of seeds from each individual plant was used as the representative value for that individual. Average values were calculated separately for seeds from early-flowering cleistogamous flowers, early-flowering chasmogamous flowers, late-flowering cleistogamous flowers, and late-flowering chasmogamous flowers. The Mann–Whitney U-test was used to compare seed traits among these groups.

Position of first flower bud formation

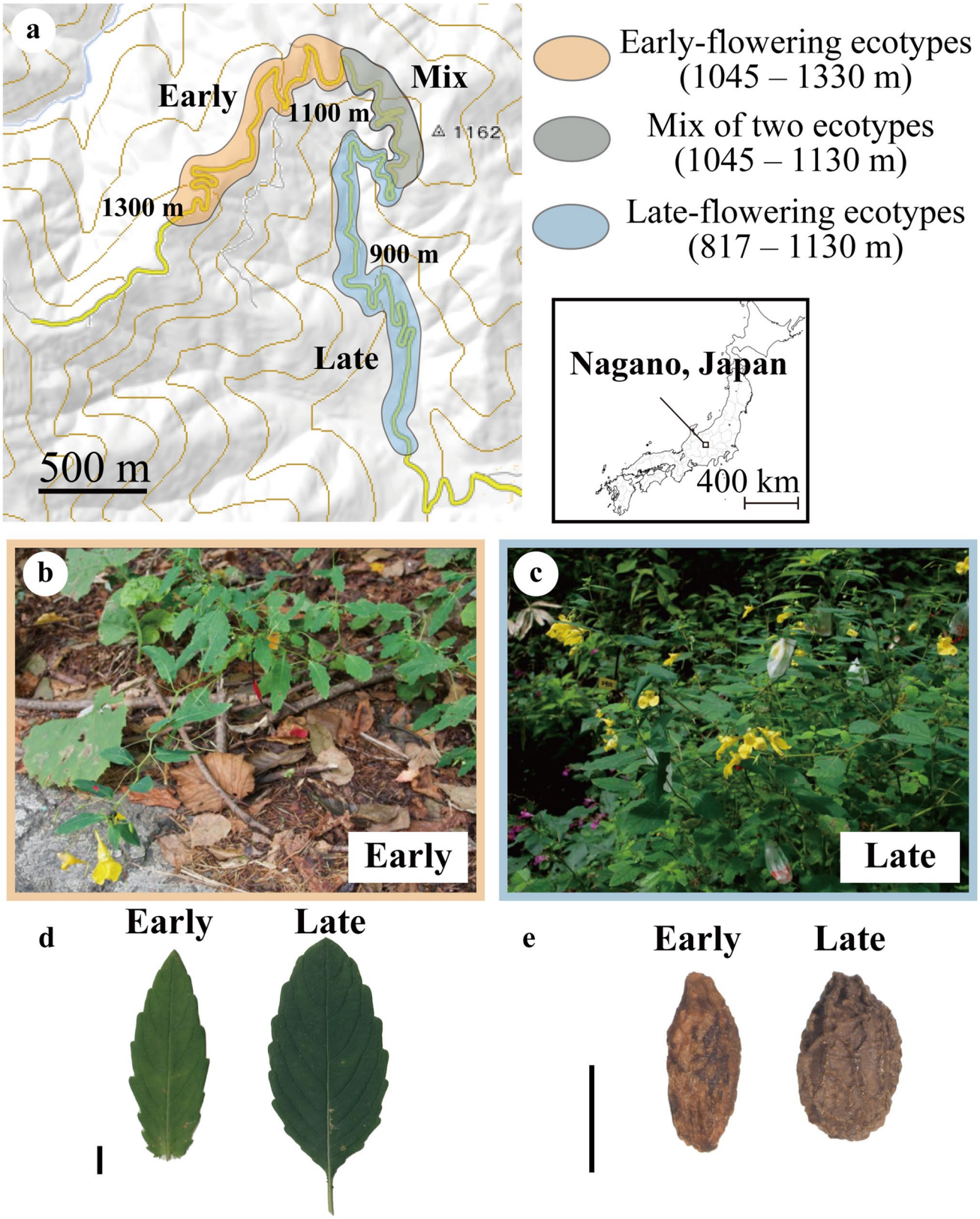
We expected that there would be differences in the vegetative growth stage at which flower buds formed between the early- and late-flowering types. We randomly collected seedlings at a similar growth stage and recorded the number of the node where the first flower bud appeared. Nodes were counted from the bottom of the plant. We conducted the investigation at sites at elevations of 1325 and 890 m, where a transplant experiment was being conducted for another research purpose.

One hundred fifty seedlings of the early-flowering type from 1325 m and 150 seedlings of the late-flowering type from 890 m were planted at intervals of 20 cm at the 1325 and 890 m transplant sites in May 2021 (so that each of the two transplant sites contained 150 seedlings of the early-flowering type and 150 seedlings of the late-flowering type). Thereafter, weekly observations were made and the number of the nodes at which buds had formed was recorded at the time that buds could first be seen on each individual. The Mann–Whitney U-test was used to compare the number of the node with the first bud between types and between populations. Individuals that died before flower bud formation and those with unknown node counts were excluded from the analysis.

Results

Plant distribution

Impatiens noli-tangere was observed at elevations between 817 and 1330 m in the Misato region of Japan (Fig. 1a). The early-flowering type (Fig. 1b) was distributed between elevations of 1045 and 1330 m, and the late-flowering type (Fig. 1c) was distributed between elevations of 817 and


a
Early
Mix
1100 m
1162
1300 m
900 m
Late
500 m
Early-flowering ecotypes
(1045 – 1330 m)
Mix of two ecotypes
(1045 – 1130 m)
Late-flowering ecotypes
(817 – 1130 m)
Nagano, Japan
400 km
b
Early
c
Late
d
Early
Late
e
Early
Late

◂**Fig. 1** Two ecotypes of *I. noli-tangere* occur in the Misato Mountain region of Nagano Prefecture, Japan. **a** An early-flowering type grows at higher elevations, and a late-flowering type grows at lower elevations. A contact zone (mixed ecotypes), where both types were observed, was found at intermediate elevations. **b** Photograph of an early-flowering type plant. **c** Photograph of a late-flowering type plant. **d** Leaf morphology of the early-flowering type (left) and late-flowering type (right). Scale bar is 1 cm. **e** Seed morphology of the early-flowering type (left) and late-flowering type (right). Scale bar is 1 mm. The map in (**a**) is based on a Digital Topographic Map published by the Geospatial Information Authority of Japan

1130 m. The early-flowering type grew on the relatively dry floor of a cedar forest growing along a mountain ridge where *Sasa* sp. plants were the dominant understory vegetation. The late-flowering type (Fig. 1c) was distributed in moist cedar forests along streams, where other herbaceous plants, including *Persicaria filiformis* (Polygonaceae), *Boehmeria silvestrii* (Urticaceae), and *Impatiens textorii* were also found. The *I. textorii* population which is known to cause reproductive interference against *I. noli-tangere* (Tokuda et al. 2015) was found along a path through the forest at elevations of 817–1130 m, which included the lower part of the *I. noli-tangere* elevational distribution range. No *I. noli-tangere* individuals with intermediate morphology were observed in this contact zone. Distribution surveys were conducted intermittently in 2013, 2015, and 2021, but the distribution ranges of the two types did not change in any of the survey years.

At the three sites in the contact zone, at elevations of 1045, 1118, and 1130 m, the two types were exclusively distributed; each formed its own patches (Fig. S1).

Flowering phenology and flower reproduction

At the contact zone at 1130 m elevation, most of the flowers produced by the early-flowering type were cleistogamous. Individuals of the early-flowering type produced at most six chasmogamous flowers (Fig. S2), and most individuals produced zero or one chasmogamous flower (Fig. S2). In contrast, all individuals of the late-flowering type produced both chasmogamous (Fig. S2) and cleistogamous flowers (Fig. S3). However, as with the early-flowering type, most of the flowers produced were cleistogamous.

The early-flowering type started flowering on June 23, whereas the late-flowering type started flowering one month later, on July 23 (Fig. 2). The start of flowering was significantly different between the two types ($n = (10, 7)$, $U = 0$, $P < 0.01$). Figure S2 shows the flowering phenology of the chasmogamous flowers of both types. Although the start date of flowering of chasmogamous flowers was not significantly different between the early- and late-flowering types ($n = (7, 7)$, $U = 13$, $P = 0.164$), the peak flowering date of chasmogamous flowers differed significantly between the two types ($n = (7, 7)$, $U = 0$, $P < 0$ 0.01). Individuals of the early-flowering type that did not produce chasmogamous flowers were excluded from this analysis.

The total number of flowers produced was significantly different between the early- and late-flowering types (comparison by Student's t-test after correction by the Bonferroni method; Fig. 3a). The early-flowering type produced 75.3–91.4 flowers on average, whereas the late-flowering type produced 344.7 flowers on average, that is, 4–5 times more flowers than the early-flowering type. The percentage of chasmogamous flowers relative to the total number of flowers produced per individual was significantly different among the surveyed populations (comparison by Mann–Whitney U-test after correction by Bonferroni's method; Fig. 3b), but no trend was observed between the early- and late-flowering types.

Leaf morphology

The leaf shape index (LL/LW) differed significantly between the early- and late-flowering types (comparison by Mann–Whitney U-test after correction by the Bonferroni method; Fig. 3c), with the early-flowering type having narrower leaves. The number of serrations also differed significantly between the early- and late-flowering types (comparison by Mann–Whitney U-test after correction by the Bonferroni method; Fig. 3d). Discriminant analysis between the two types based on leaf morphology (leaf length, leaf width, and number of serrations) was accurate about 98% of the time. The number of serrations contributed significantly to the first canonical discriminant-function coefficients (scoring coefficient of serrations, $P < 0.001$, $F = 221.699$). These results indicate that leaf traits are a good indicator that can be used to distinguish between the early- and late-flowering types before and after the flowering period.

Seed morphology

Seeds of the early-flowering type were longer and narrower and lighter colored than seeds of the late-flowering type (Fig. 1e). The seed length/width ratio did not differ between cleistogamous and chasmogamous flowers of the same type (Fig. 3e; early-flowering type cleistogamous flowers vs. chasmogamous flowers: $n = (15, 15)$, $U = 80$, $P > 0$ 0.05; late-flowering type cleistogamous flowers vs chasmogamous flowers: $n = (14, 16)$, $U = 68$, $P > 0$ 0.05). However, the seed length/width ratio (seed length/seed short diameter) of seeds derived from both chasmogamous and cleistogamous flowers was significantly different between early-flowering and late-flowering types (Fig. 3e; cleistogamous flowers early-flowering type vs late-flowering type: $n = (15, 14)$, $U = 0$, $P < 0.01$; chasmogamous flowers early-flowering type vs late-flowering type: $n = (15, 16)$, $U = 0$, $P < 0.01$).

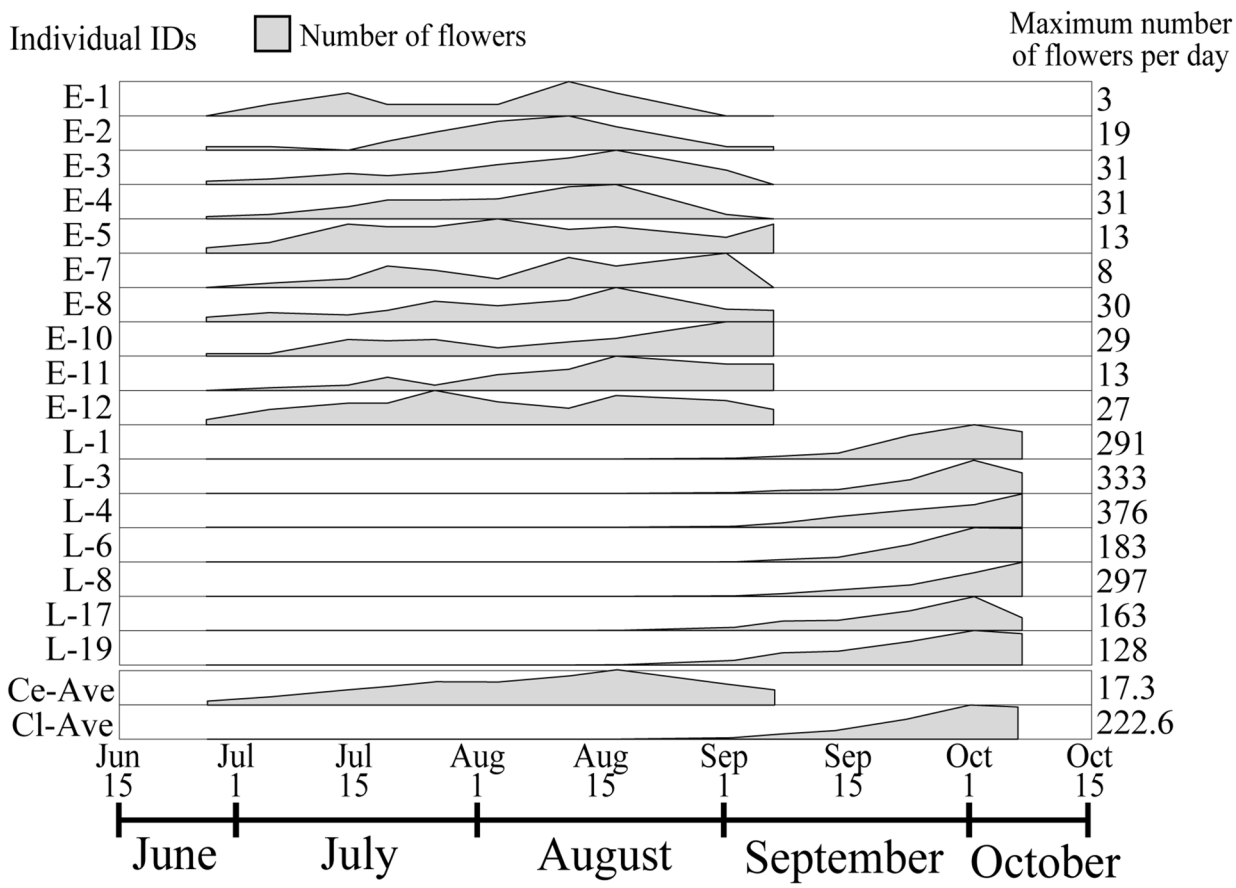


Fig. 2 Flowering phenology of each surveyed individual of *I. noli-tangere* at the contact zone (1130 m). The sum of chasmogamous and cleistogamous flowers is shown; see Figs. S2 and S3 for separate count data for chasmogamous and cleistogamous flowers, respectively. The identification number of each individual is shown on the left vertical axis (E indicates early flowering, and L indicates late flowering). Individuals that died before flowering have been excluded. The maximum number of the flowers is shown on the right vertical axis. The mean numbers of flowers of early- and late-flowering types (E-Ave and L-Ave, respectively) are shown at the bottom. The horizontal axis shows the date

Position of first flower bud formation

There was a significant difference in the number of the node of first flower bud formation between the early- and late-flowering types ($n=(144, 145)$, $U=3.5$, $P<0.01$; Fig. 3f). The early-flowering type had buds from at least the fourth node, and the average number of the node with the first flower bud was 5.46. The late-flowering type had buds from at least the ninth node, and the average number of the node with the first flower bud was 14.13. The late-flowering type produced buds only after sufficient branching had occurred, whereas the early-flowering type produced buds directly from the nodes on the plant stem.

Discussion

We found that early- and late-flowering ecotypes of *I. noli-tangere* grow in sympatry in the Misato region of Japan, with differentiation in flowering phenology being consistently maintained. In addition to the different flowering phenology (Fig. 2), as reported by Masuda and Yahara (1994), we found large differences in leaf and seed morphology, the flower bud formation pattern, and the number of flowers produced (Fig. 3). Masuda and Yahara (1994) have already shown that flowering phenology in *I. noli-tangere* is strongly heritable. In this study, the early-flowering type of *I. noli-tangere* formed flower buds at lower nodes, before the plants had reached their full growth, whereas the late-flowering type formed flower buds at higher nodes, when the plants were more mature (Fig. 3f). The variation in flowering phenology may reflect this difference in the position of the nodes where the flower buds form.

Variation of flowering phenology is common in many plant species, and continuous variation along an environmental gradient such as latitude or elevation often occurs (Alexander et al. 2009; Fabbro and Korner 2004; Jonas and Geber 1999; Korner 1999). Shinohara et al. (2015) conducted a study of *I. noli-tangere* along an elevational

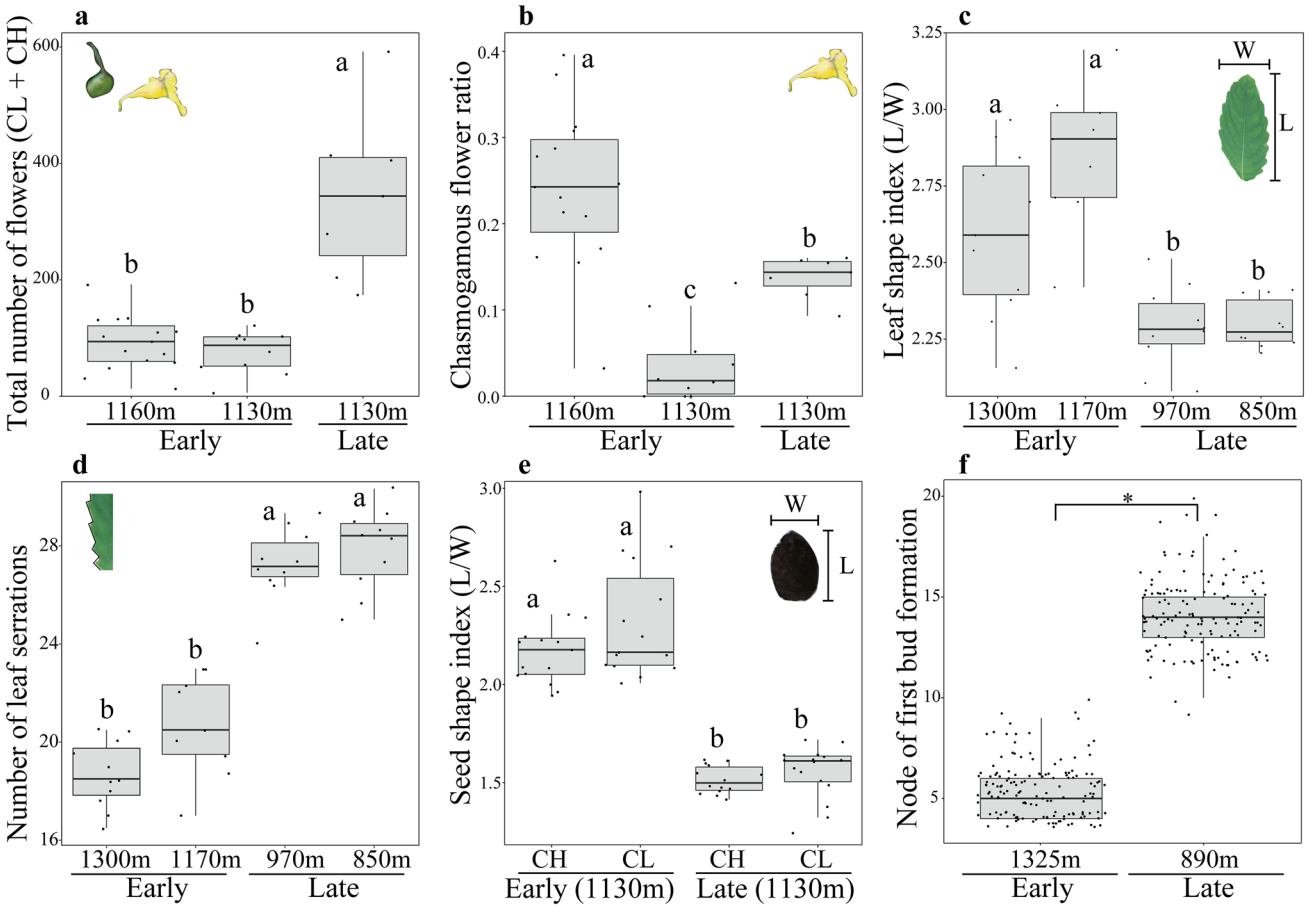


Fig. 3 Differences in plant traits between early-flowering and late-flowering types of *I. noli-tangere*. The surveyed elevation and the ecotype (Early or Late) of *I. noli-tangere* are shown on the horizontal axis. **a** Total flower production (chasmogamous (CH) + cleistogamous (CL) flowers). **b** Ratio of chasmogamous flowers to all flowers produced. **c** Leaf shape index (L/W aspect ratio). **d** Number of serrations on leaves. **e** Seed shape index (L/W aspect ratio). **f** Number of the node of first flower bud formation on the ramet. Different letters and asterisks in each panel indicate statistically significant differences (after correction by the Bonferroni method). The total numbers of flowers were compared by Student's t-test; other comparisons were made by the Mann–Whitney U-test. Dots in all box plots indicate actual data plots

gradient and observed not a continuous variation of flowering phenology but the discontinuous distribution of early- and late-flowering types. Their study revealed that the two types of flowering phenology were clearly distinguishable even at contact zones between the early-flowering and late-flowering types. The early-flowering type was distributed along a ridge, and the late-flowering type was distributed in the relatively wetter environment of a valley (Fig. 1a); the environment of the contact zone between the two types was intermediate. This exclusive distribution of the two types at the contact zone suggests that some kind of reproductive isolation mechanism may exist between the two distinct types.

The two ecotypes of *I. noli-tangere* were roughly distributed at different altitudes. The plant distribution in the contact zone was structured such that each ecotype was clustered (Fig. 1S). These patterns may reflect environmental differences between the higher and lower altitudes. The differences in their sympatric distribution may reflect differences in microhabitat (e.g., Palgunadi et al. 2021). Ecotype differentiation along elevation has been rarely reported (reviewed in Itino and Hirao 2016). For example, *Cimicifuga simplex* has three ecotypes distributed at different altitudes, each with a different reproductive system and different pollinators (Pellmyr 1986; Toji and Itino 2020; Toji et al. 2020). Here, we report that *I. noli-tangere* also shows ecotype differentiation along elevation. These two research systems may be useful for examining the evolutionary pattern of intraspecific altitudinal differentiation, a topic which has not yet been well investigated. They can also contribute to the formulation of countermeasures against the loss of ecotype diversity due to the narrowing of elevational distributions (Freeman et al. 2018) caused by future global warming.

This study found differences in leaf and seed traits between early- and late-flowering types of *I. noli-tangere*. The

early-flowering type had narrower, entire leaves, while the late-flowering type had wider, serrated leaves (Figs. 1d, 3d). The early-flowering type had lighter-colored and thinner seeds than the late-flowering type (Figs. 1e, 3e). The habitat differences may impact the leaf and seed traits and affect photosynthetic capacity and germination rate (Liu et al. 2022; Luzuriaga et al. 2006; Mitsui et al. 2010; Setoguchi and Kajimaru 2004; Tsukaya 2002; Yates et al. 2010). Therefore, measuring the environmental conditions in which two ecotypes grow is a needed future task.

Impatiens pallida, which is closely related to *I. noli-tangere*, blooms early on the forest floor, where the density of herbivorous insects is high, but blooms late at the forest edge, where the density of herbivorous insects is low (Schemske 1984). In *I. pallida*, the date of onset of cleistogamous flower bud formation is genetically fixed in each population and is a key trait for differentiating ecotypes in this species (Bennington and McGraw 1995). In other regions of Japan, *I. noli-tangere* has been reported to suffer feeding damage from herbivorous sawfly larvae from July to September (Suzuki et al. 2007). One limitation of this study is that the selective pressures acting on trait differentiation in *I. noli-tangere* have not been fully elucidated. In addition, chasmogamous flowers of *I. noli-tangere* bloom only about three days, so accurate flower counts require observations over a much shorter period than every week, such as in our study. In the future, we plan to test whether environmental differences between higher and lower elevations exert different selective pressures that lead to flowering differentiation by measuring environmental gradients in the study area and by conducting a reciprocal transplant experiment.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10265-023-01444-w.

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Data availability The data supporting the present study's findings are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest There are no conflicts of interest.

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