Balsaminaceae in Southeast Asia: systematics, evolution, and pollination biology

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CHAPTER 7

The evolution of pollination syndromes and corolla symmetry in *Impatiens* (Balsaminaceae)

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7.1. ABSTRACT

Floral diversity may evolve as a result of plant-pollinator interactions by two distinct processes: shifts between functional pollinator groups, or divergent use of the same pollinator. However, the frequency and relative importance of these processes are rarely considered simultaneously. Here we apply a phylogenetic approach using the recently radiated, large plant genus *Impatiens*, characterized by profound variation in corolla shape and colour (indicative of the use of different pollination systems), and corolla symmetry (indicative of the divergent use of the same pollinator), to understand the association between pollination and floral evolution. We reviewed pollination studies on *Impatiens* to predict pollination systems for a large number of *Impatiens* species represented in the largest phylogenetic analysis of the genus to date. Corolla symmetry of each species was recorded from fresh specimens, photographs, and literature. The evolution of pollination systems and floral symmetry was reconstructed using Parsimony and Maximum Likelihood criteria, using a combined phylogenetic analysis of ITS and *atpB-rbcL* DNA sequence data, with a focus on Asian species (251 from 281 samples). *Impatiens* species are pollinated by bees, a combination of bees and Lepidoptera, birds, flies, or are autogamous. Based on associations between floral morphology and known pollination systems, 71% of studied species was inferred to be bee-pollinated, 22% bimodally pollinated, 3% bird-pollinated, and 5% autogamous or fly-pollinated. Nineteen percent of the species have an asymmetrical corolla, but this character state only occurs among bee-pollinated species. Ancestral state reconstructions showed that bee pollination and zygomorphic flowers are ancestral in the genus and family. The ancestral state of corolla symmetry of *Impatiens* and Balsaminaceae is strongly influenced by the state assigned to the polymorphic species *Hydrocera triflora*. The average number of pollination system shifts is around 40 times (14% of maximum possible changes) and shifts were concentrated in a clade of the *I.* sect. *Uniflorae* which includes some African and Madagascan species. The average number of corolla symmetry shifts is around 20 times (7%) and was concentrated in other clades that were predominantly bee-pollinated and mainly distributed in Asia. Although parsimony reconstructions suggest a larger number of shifts towards asymmetry, a model with a single rate was not significantly worse than a model with separate forward and backward rates. Floral evolution in *Impatiens* occurs through both pollination system shifts and divergent use of the same pollinator, although the former appears more frequent. These processes may be driven by the availability of pollinator species at large geographical scales. However, the overall frequency of

both processes only explains a relatively small percentage of cladogenic events, suggesting that other processes may also have driven floral evolution.

7.2. INTRODUCTION

Floral diversity is traditionally associated with pollinator driven evolutionary processes (Stebbins, 1970; Grant, 1949, 1994; Grant & Grant, 1965; Darwin, 1862). Two distinct processes have been identified. First, populations may adapt to different functional pollinator groups resulting in the formation of pollination ecotypes. During this process, populations diverge in floral traits, which reflects the sensory bias and morphological differences between different locally most effective pollinators (Johnson, 1997; Robertson &Wyatt, 1990; Johnson *et al.,* 1998). This process is driven by geographical turnover in pollinator species (Van der Niet *et al*., 2014b; Johnson & Steiner, 1997; Johnson, 1997). Second, populations may not adapt to a different pollinator but diverge in the way a particular pollinator is utilised (Armbruster *et al*., 1994; Waterman *et al*., 2011 Eaton *et al*., 2012). This process is mostly associated with divergence in floral traits which mediate the site of pollen placement on the pollinator, and is thought to be driven by local competition for pollinator services. Pollinator-driven evolutionary processes are best studied at the population level. Indeed, several studies show the adaptation of flower traits to pollinator both within populations or species (Boberg *et al*., 2014; Gómez *et al*., 2014; Cosacov *et al*., 2014; Peter & Johnson, 2014; Van der Niet *et al*., 2014b).

While species-specific studies are important for understanding evolutionary processes, they fail to identify their relative importance for lineage diversification. This requires a comparative perspective in a phylogenetic context. In particular, reconstructing the evolution of pollination systems by quantifying the frequency and direction of shifts in pollination system, and divergent use of the same pollinator can be done in plant groups with diverse floral characters, sufficient pollination data, and an adequately sampled phylogeny. Although many studies have assessed the frequency and direction of shifts in a pollination system (reviewed in Van der Niet & Johnson, 2012), and few studies have considered the frequency of shifts in use of the same pollinator (e.g. Eaton *et al*., 2012), no macroevolutionary analysis has been carried out so far that quantified the overall frequency of pollinator-driven evolution and the relative importance of both pollination system shifts and the divergent use of the same pollinator.

Impatiens, a genus with more than 1000 species, is characterized by great floral diversity (Grey-Wilson, 1980). The architecture of *Impatiens* flowers comprises various non-actinomorphic perianth parts and the stigma that is receptive after anther shedding (Grey-Wilson, 1980). The floral morphology of balsams includes a nectar-producing spur as part of the lower sepal. Comparative studies have revealed that variation in size and shape of the perianth parts is associated with predictable differences in pollination systems (Ruchisansakun *et al*., 2016; Grey-Wilson, 1980; Abrahamczyk *et al*., 2017; Kato *et al.* 1991). Besides the presence of distinct pollination syndromes, species which share the same pollinator also vary in floral architecture. In particular, floral variation among these species is associated with precise placement of pollen on the pollinator bodies, as was confirmed independently among co-flowering bee-pollinated species in Asia, and bird-pollinated species in Africa (Janeček *et al*., 2015; Ruchisansakun *et al*., 2016). In several cases differential pollen placement is achieved by a highly unusual mechanism of floral asymmetry in which the lower lateral petals are asymmetrical. Based on the two distinct types of floral variation in association with different pollination systems and divergent use of the same pollinator, *Impatiens* is an ideal group to evaluate the overall frequency and relative importance of two distinct processes of pollinator-driven evolution.

The current study therefore aims to reconstruct the evolution of pollination systems and divergent use of the same pollinator in *Impatiens*. First we compiled all pollination studies of *Impatiens* species and used this to set up a predictive framework for inferring pollinators for species sampled in the largest phylogenetic analysis of the genus to date. Together with data on floral symmetry, we used the phylogenetic tree for a comparative analysis and ancestral character state reconstruction.

7.3. METHODS

7.3.1. Phylogenetic Analyses

Taxon sampling

To reconstruct the evolution of pollination systems and corolla symmetry of *Impatiens,* focusing on Asian species, all Balsaminaceae species for which both ITS and *atpB-rbcL* sequences are available were included, consisting of 281

Impatiens (ca. 25% of all species in the genus) and *Hydrocera triflora*, the monospecific sister-genus of *Impatiens*. Among the sampled *Impatiens* samples, 251 are from Asia, while 30 species are from Africa, Europe, Madagascar, and North America. The analysis is therefore biased towards Asian species. This region comprises three out of the five informal hotspots of *Impatiens* diversity and the pollination ecology of these species is arguable the most well-understood. *Marcgravia umbellata* is used as an outgroup.

DNA sequencing

To reconstruct the phylogenetic tree for *Impatiens*, DNA sequences from plastid and nuclear genomes were used as characters. Most DNA sequences used for phylogeny reconstruction were mined from Genbank (Yu *et al*., 2015; Ruchisansakun *et al*., 2015; Utami & Ardiyani, 2015; Shajitha 2016a, 2016b, Yuan *et al*., 2014), but for 27 species DNA sequences were newly generated. For these species, genomic DNA was extracted from fresh or silica-dried leaf material and herbarium specimens using a CTAB protocol (Doyle & Doyle, 1987). The nuclear ribosomal internal transcribed spacer ("ITS" hereafter) and the plastid *atpB-rbcL* intergenic spacer ("*atpB-rbcL*" hereafter) were amplified following the protocols of Yuan *et al.* (2004) and Janssens *et al.* (2006) respectively. Standard DNA sequencing using the original amplification primers, was done by Macrogen (Amsterdam, the Netherlands). The chromatograms of forward and reverse sequences were combined by De Novo Assemble to create contigs in Geneious 10.2.2 (Biomatters Ltd, New Zealand). Sequences were aligned using MUSCLE alignment and manually edited in Geneious 10.2.2 (Biomatters Ltd, New Zealand).

To obtain a set of fully resolved, ultrametric trees for ancestral character state reconstruction, Bayesian Evolutionary Analysis Sampling Trees analysis was implemented using BEAST v.1.8.4 (Drummond & Rambaut, 2007), run on the Cipres platform. All parameters for the BEAST analysis were set in BEAUTI v.1.8.0 (Drummond & Rambaut, 2007) as described below. Based on the Akaike Information Criterion test as implemented in jModelTest2 v0.1.1 (Darriba *et al*., 2012), the GTR+I+G model was chosen for ITS whereas the GTR+G model was selected for *atpB-rbcL*. Base frequencies were set to "estimate" and "4 rate categories". First, combined and separated (ITS and *atpB-rbcL*) trees were reconstructed and used for character state reconstruction and investigating the frequency and direction of evolution (see below). We decided to use the combined data tree as the main tree in our analysis to represent both genes, despite significant incongruence between the two gene trees. We implemented the Lognormal relaxed clock model, enforcing the “estimate” option in the clock model. As Tree Prior, we used a Birth-Death Incomplete Sampling process (Stadler, 2009) with a Random starting tree. To explore parameter and tree space, we set the length of the Markov chain Monte Carlo (MCMC) chain to 10000000. All other settings were according to the default settings. The Maximum clade credibility trees (MCC) was constructed in TreeAnnotator v1.8.0, burn in 1000, posterior probability limit 0.5.

7.3.2. Character Evolution

Pollination systems

For most *Impatiens* species included in the phylogenetic analysis, pollination systems are unknown. However, given the strong associations between certain floral traits (floral entrance width, spur length and petal colour), and pollination systems (Grey-Wilson, 1980; Ruchisansakun *et al*., 2016; Abrahamczyk *et al*., 2017), these floral traits of species for which direct field-based observations are lacking can be used to infer their pollination systems.

To infer the pollination system of *Impatiens* species that were not studied in the field, we reviewed all literature related to *Impatiens* pollination and assessed which floral characters were associated with particular pollination systems. We specifically focused on the following floral characters that were identified in previous comparative studies (Grey-Wilson, 1980; Ruchisansakun *et al*., 2016; Abrahamczyk *et al*., 2017): floral entrance width (small vs large), spur length (short vs long) and petal colour. Pollination systems were inferred according to functional groups including bees, birds, Lepidoptera, flies, as well as autonomous self-pollination.

Corolla symmetry

To assess corolla symmetry (i.e. zygomorphic vs asymmetrical flowers according to the different size or shape of lateral petals) we analysed fresh material, investigated photographs taken by the authors, or photographs in books or on websites which were identified by *Impatiens* specialists, studied illustrations and descriptions in the literature, and examined dried specimens or illustrations of herbarium specimens. Each species was classified as either zygomorphic or asymmetrical. *Hydrocera* is polymorphic for corolla symmetry in which the zygomorphic form is more common than the asymmetrical form. However, given the potentially large effect of the character state of *Hydrocera* on ancestral character state reconstructions due to its phylogenetic position, an analysis to assess the sensitivity of the results to the coding of *Hydrocera* was carried out by running all ancestral character state reconstructions with both codings.

Character state reconstructions

To reconstruct the ancestral state, frequency, and direction of evolution of pollination systems and corolla symmetry, we used Fitch parsimony (Fitch, 1971) and maximum likelihood ancestral character state reconstruction methods implemented in Mesquite (Maddison & Madison, 2018).

The position of shifts was reconstructed based on a set of 10,000 BEAST trees, the first 1,000 burnin trees were ignored. For the Likelihood ancestral state reconstruction of the multi-state character ‘pollination system’, only the one-parameter Markov k-state (Mk1) model was used (Lewis, 2001). The transition rate between pollination systems were estimated. For reconstructing the evolution of the binary character corolla symmetry, we used an Asymmetry Likelihood Ratio Test (ALR test) to choose whether the Mk1 model performs significantly worse than AsymmMk model (Pagel, 1999; Ree & Donoghue, 1999). For the ALR Test, the MCC tree was used in combination with the corolla symmetry data matrix in “Tree value using character” in Mesquite.

To summarize the frequency and direction of evolution, we used the function “state change over trees” in Mesquite, using parsimony. To determine the relative frequency of shifts, the percentage of the shifts was calculated by dividing the number of shift by the maximum number of possible shifts on the tree (number of taxa - 1).

7.4. RESULTS

7.4.1. Inference of pollination systems and corolla symmetry

Data on pollination systems were available for 54 *Impatiens* species (Table 7.1). The majority of studies was done on Asian species (N=32), followed by African species (N=10), European species (N=8), and American species (N=4). Most *Impatiens* species are pollinated by more than one pollinator species. Apart from African species, the majority of species are bee-pollinated. Furthermore, most species pollinated by Lepidoptera are also pollinated by bees (Table 7.1).

Based on the association between floral characters and pollinations systems, for all Asian, European, and North American species, the results of the literature survey were used to classify them as follows: species with a short spur and large floral chamber were inferred to be bee-pollinated; species with long spur and small floral chamber were inferred to be pollinated by both Lepidoptera and bees (bimodal pollination system). Species with small, spurless flowers were inferred to be autogamous or fly-pollinated (Table 7.1). Given the paucity of pollinator observations for the latter category, we cannot distinguish between autogamy and fly-pollination in our results (see below). Besides the pollination systems outlined above, an additional system for African species was used for the species with a large floral entrance, short spur, and red flowers; these were inferred to be bird-pollinated.

Among the *Impatiens* species sampled, 200 species are considered bee-pollinated (70.9 %), 61 species pollinated by Lepidoptera and bees (21.6 %), 14 species are autogamous or pollinated by flies (5.0 %), and 7 species are bird-pollinated (2.5 %). Furthermore, 51 species (18.5%) have an asymmetrical corolla.

Table 7.1. List of studied species, study sites, and pollinators.

***Impatiens* species (*species in the Fig. 7.1 & 7.S1)**	**Study sites (*non-native)**	**Floral morphology**			**Pollinators**	**References**
		Floral entrance width	**Spur length**	**Petal colour**		
			Africa			
I. burtonii var. *burtonii**	Cameroon	Large	Short	Pink	**Bees:** *Apis mellifera* **Flies:** *Rhingia mecyana*, *Melanostoma* sp.	Vlašánková *et al*., 2017
I. etindensis	Cameroon	Large	Short	Red	**Birds:** *Cyanomitra oritis*, *Creich, Cinnyris reichenowi*	Janecek *et al*., 2015
I. frithii	Cameroon	Large	Short	Red	**Birds:** *Cyanomitra obscura*, *Cyanomitra oritis*, *Cinnyris johannae*	Janecek *et al*., 2015; Bartoš & Janecek, 2014
I. grandisepala	Cameroon	Large	Short	Red	**Birds:** *Cyanomitra obscura*	Janecek et al., 2015
I. hians	Cameroon	Large	Short	Red	**Birds:** *Cinnyris ursulae*, *Cyanomitra obscura*, *C. oritis*	Janecek et al., 2015

Impatiens **species (*species in the Fig. 7.1 & 7.S1)**	**Study sites (*non-native)**	**Floral morphology**			**Pollinators**	**References**
		Floral entrance width	**Spur length**	**Petal colour**		
Africa						
I. hochstetteri	South Africa	Small	Long	White	**Flies:** *Stenobasipteron wiedemanni*	Potgieter & Edwards, 2005
					Lepidoptera: *Papilio echerioides*, *P. nireus*, *P. demodocus*	Vogel., 1954
*I. kilimanjari**	Africa	Large	Short	Red	**Birds:** *Cyanomitra olivacea*, *Nectarinia mediocris*	Abrahamczyk *et al*., 2017
*I. niamniamensis**	Cameroon	Large	Short	Red	**Birds:** *Cyanomitra oritis*, *C. alinae*	Janecek *et al*., 2015
I. pinganoensis	Angola	Small	Long	White	**Flies:** *Empis* sp.	Abrahamzyk *et al*., 2016
*I. sakeriana**	Cameroon	Large	Short	Red	**Birds:** *Cyanomitra oritis*, *Creich, Cinnyris reichenowi*	Janecek et al., 2015
America						
I. biflora	USA	Large	Short	Orange	**Bees:** *Bombus vagans*, *Bombus impatiens*, **Birds:**	Schemske, 1978
I. capensis *	USA	Large	Short	Orange	**Bees:** Bumblebee	Wilson, 1995
					Bees: *Bombus vagans*	Rust, 1977
I. pallida	USA	Large	Short	Yellow	**Bees:** *Bombus vagans*	Rust, 1977
					Bees: *Bombus vagans, Bombus impatiens*	Schemske, 1978
					Bees: Bumblebee	Wilson, 1995
Asia						
*I. campanulata**	India	Small	Long	Pale yellow	**Bees:** *Apis cerana* *Trigona iridipennis* **Lepidoptera:** *Macroglossum corythus* *M. variegatum* Other utterflies	Sreekala *et al*., 2008a Kulloli *et al*., 2009b
*I. chiangdaoensis**	Thailand	Small	Long	Pink	**Bees:** *Bombus haemorrhoidalis, Apis cerana* **Lepidoptera:** *Notocrypta curvifascia*	Ruchisansakun *et al*., 2016
I. coelotropis	India	Large	Short	Red and green	**Bees:** *Apis cerana* *Apis dorsana* *Trigona sp.* **Lepidoptera:** *Macroglossum corythus* *M. variegatum* Lepidoptera	Sreekala *et al*., 2008b

***Impatiens* species (*species in the Fig. 7.1 & 7.S1)**	**Study sites (*non-native)**	**Floral morphology**			**Pollinators**	**References**
		Floral entrance width	**Spur length**	**Petal colour**		
Asia						
I. cuspidata *	India	Small	Long	Pink	**Bees:** *Apis cerana* *Trigona sp.* **Lepidoptera:** *Macroglossum variegatum* *M. corythus* Lepidoptera **Flies**	Sreekala *et al*, 2011
I. dalzelii *	India	Large	Short	Yellow	**Bees:** *Apis florea, Apis indica, Trigona iridipennis*	Kulloli & Sreekala, 2009
*I. daraneenae**	Thailand	Large	Short	Pink	**Bees:** Apidae: unknown sp.	Ruchisansakun *et al*., 2016
I. eubotrya	Indonesia	Large	Long	Yellow	**Bees:** *Amegilla sumatrana*, *Amegilla andrewsi*, *Elaphropoda impatiens*	Kato *et al*., 1991
*I. gardneriana**	India	Small	Long	Pink	**Bees:** *Apis cerena*, *Apis dorsata*, *Apis indica*, *Trigona iridipennis* **Lepidoptera:** *Macroglossum variegatum*, Pacl	Kulloli *et al*., 2010
*I. grandis**	India	Small	Long	White and red	**Bee:** *Apis cerana* *Trigona iridipennis* *Apis dorsata* **Lepidoptera:** *Parantica aglea, Badamia exclamationis, Rpthima baladus, Macroglossum stellatarum, M. corythus, Euploea core, etc.*	Kulloli *et al*., 2015
*I. henslowiana**	India	Small	Long	Pink	**Bees:** *Apis florea, Apis indica* **Lepidoptera**	Sreekala *et al*., 2007
I. hypophylla	Japan	Large	Short	White	**Bees:** *Bombus diversus*	Kato, 1988
*I. kerriae**	Thailand	Large	Short	White	**Bees:** *Bombus haemorrhoidalis*	Ruchisansakun *et al*., 2016
I. korthalsii	Indonesia	Small	Short	Yellow	**Bees:** *Thrinchostoma asianum, Amegilla sumatran, A. andrewsi,* *Elaphropoda impatiens*	Kato *et al*., 1991
I. leptura	India	Small	Long	Pink	*Honeybee, lepidoptera, hawkmoths*	Sreekala *et al*., 2013
*I. maculata**	India	Small	Long	Pink	**Bees:** *Apis cerena, A. dorsata, Trigona* sp. **Butterfies:** *Badamia exclamationis*, *Papilo demoleus*, *Pachiopta aristolochiae*, *Danaus genutia*	Sreekala & Kulloli, 2014

Impatiens **species (*species in the Fig. 7.1 & 7.S1)**	**Study sites (*non-native)**	**Floral morphology**			**Pollinators**	**References**
		Floral entrance width	**Spur length**	**Petal colour**		
Asia						
*I. muscicola**	Thailand	Large	Spurless	White to pink	**Autogamy**	Ruchisansakun *et al*., 2016
I. noli-tangere *	Japan	Large	Short	Yellow	**Bees:** *Bombus diversus*	Kato, 1988
I. oxyanthera *	China	Large	Short	Pink	**Bee:** *Bombus trifasciatus* Smith, *Amegilla pseudobomboides*, *Habropoda* sp.	Wang *et al*., 2013
*I. phoenicea**	India	Large	Long	Red	**Bee:** Apis cerena indica Apis dorsata Mycalesis mineus **Lepidoptera:** Ypthima baldus Hasora chromus Badamia exclamtionis Delias eucharis	Ramasubbu *et al*., 2009
I. platyadena	India	Large	Short	Red	**Bees:** *Apis cerana*, *A. dorsata indica, Trigona iridipennis* **Lepidoptera:** *Danaus chrysippus, D. genutia, Tirumala limniace, Parantica aglea, Caprona ransonnetti*	Ramasubbu *et al*., 2011
I. platypetala *	Indonesia	Small	Long	Pink	**Bees:** *Amegilla andrewsi* **Lepidoptera:** *Macroglossum corythus*	Kato *et al*., 1991
*I. psittacina**	Thailand	Large	Short	Pink	**Bees:** *Bombus haemorrhoidalis*	Ruchisansakun *et al.*, 2016
*I. pulcherima**	India	Small	Long	Pink	**Bees:** *Apis cerana, A. florea, Trigona iridipennis*	Sreekala, 2016
*I. racemosa**	Thailand	Large	Short	Yellow	**Bees:** *Bombus haemorrhoidalis*	Ruchisansakun *et al.*, 2016
I. reptans	China: Hunan	Large	Long	Yellow	**Bees:** *Apis cerena, Bombus trifasciatus,* **Lepidoptera:** *Macroglossum variegatum, M. corythus, Papilio memnon*	Tian *et al*., 2004
*I. rufescens**	India	Large	Short	Pink	**Bees:** *Apis cerena, Carpenter bee*	Mohandass, 2013
*I. santisukii**	Thailand	Small	Long	Pink	**Bees:** *Bombus haemorrhoidalis* **Lepidoptera:** *Polytremis discreta discrete, Polytremis lubricans lubricans*	Ruchisansakun *et al.*, 2016

***Impatiens* species (*species in the Fig. 7.1 & 7.S1)**	**Study sites (*non-native)**	**Floral morphology**			**Pollinators**	**References**
		Floral entrance width	**Spur length**	**Petal colour**		
Asia						
I. talangertis	Indonesia	Large	Short	Yellow	**Bees:** *Elaphropoda impatiens*	Kato *et al.*, 1991
I. textorii	Japan	Large	Short	Pink	**Bees:** *Bombus diversus*	Kato, 1988
I. trichocarpa	India	Large	Short	Pink	**Bees:** *Apis florea, A. indica*	Kulloli *et al.*, 2009a
I. verticillata	India	Small	Long	Red	**Bees:** *Apis dorsata* **Lepidoptera:** *Papilio demoleus*	Sreekala & Pandurangan, 2012
Europe						
*I. arguta**	Germany*	Large	Short	Pink to blue	**Bees:** *Bombus pascuorum*	Abrahamczyk *et al.*, 2017
I. balfourii	Ireland*	Large	Short	Pink	**Bees:** *Apis mellifera, Bombus hortorum, B. pascourum*	Ugoletti *et al.,* 2013
*I. balsamina**	Germany*	Large	Long	Pink	**Bees:** *Apis sp., Bombus hortorum, B. terrester, Polistes gallica*	Knuth, 1898
I. bisaccata	Germany*	Large	Short	Pink	**Bees:** *Pachymelus unicolor, P. bicolor, P. cambouii*	Erpenbach, 2006
*I. glandulifera**	Ireland*	Large	Short	Purple	**Bees:** *Bombus pascuorum, Bombus hortotum*	Nienhuis *et al.*, 2009
	Ireland*	Large	Short	Pink	**Bees:** *Apis mellifera, Bombus hortorum, B. pascourum*	Ugoletti *et al.,* 2013
	Germany*	Large	Short	Pink	**Bees:** *Apis mellifera , Bombus agrorum, B. lapidariu, B. terrestris*	Knuth, 1898
*I. latifolia**	Germany*	Small	Long	Pink	**Lepidoptera**	Knuth, 1898
*I. noli-tangere**	Germany	Large	Short	Yellow	**Bees:** *Bombus lapidaries, B. hortorum, B. terrester,Halictus cylindricus,H. zonulus* **Wasps:** *Vespa media*	Knuth, 1898
*I. parviflora**	Germany*	Large	Spurless	Yellow	**Flies:** *Syrphus balteatus*	Knuth, 1898

7.4.2. Phylogenetic analysis

The combined dataset has 2703 bp, 1269 bp for ITS region and 1434 bp for the *atpB-rbcL* region. The BEAST MCC tree from the combined dataset shows some incongruences with the trees from separated ITS or *atpB-rbcL* data. However, the majority of the results does not conflict to the main results from the combined dataset (Table 7.2-7.5). *Impatiens* can be divided into 8 clades (Fig. 7.1, 7.S1). Only in two clades African species were found: most are part of the big

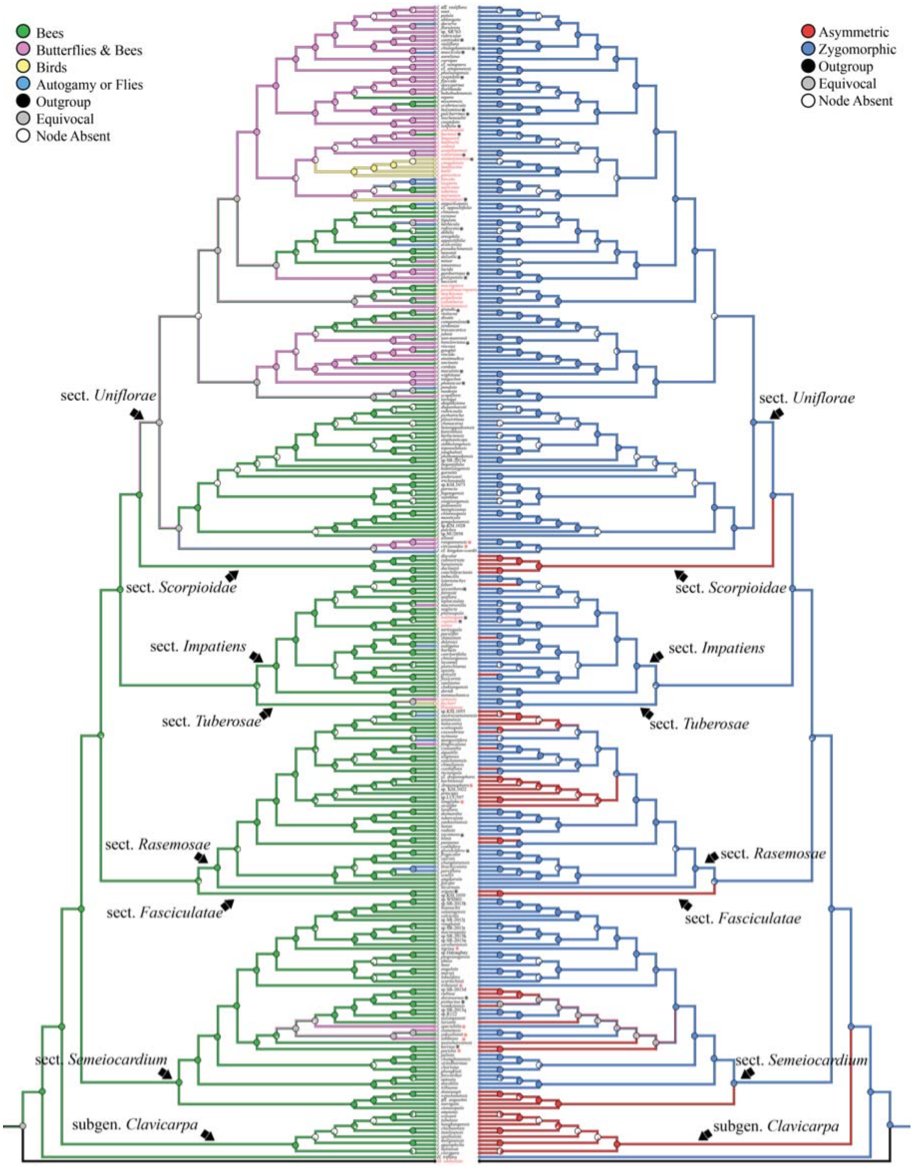

Fig. 7.1. Parsimony Ancestral character state reconstruction of pollination systems (left) and corolla symmetry (right). Branch colors represent the most parsimonious ancestral character states reconstructed using the maximum clade credibility tree. Pie charts at nodes represent the proportion of BEAST trees for which a particular character state at a node was reconstructed, the node was absent, or reconstruction was equivocal, in case that ***H. triflora* was treated as zygomorphic corolla**. The species outside Asia were marked by red alphabets. The black asterisk (*) show the species with pollination studies in the table 1. The red Asterisk (*) shows the species with pollinator were observed by Saroj Ruchisansakun.

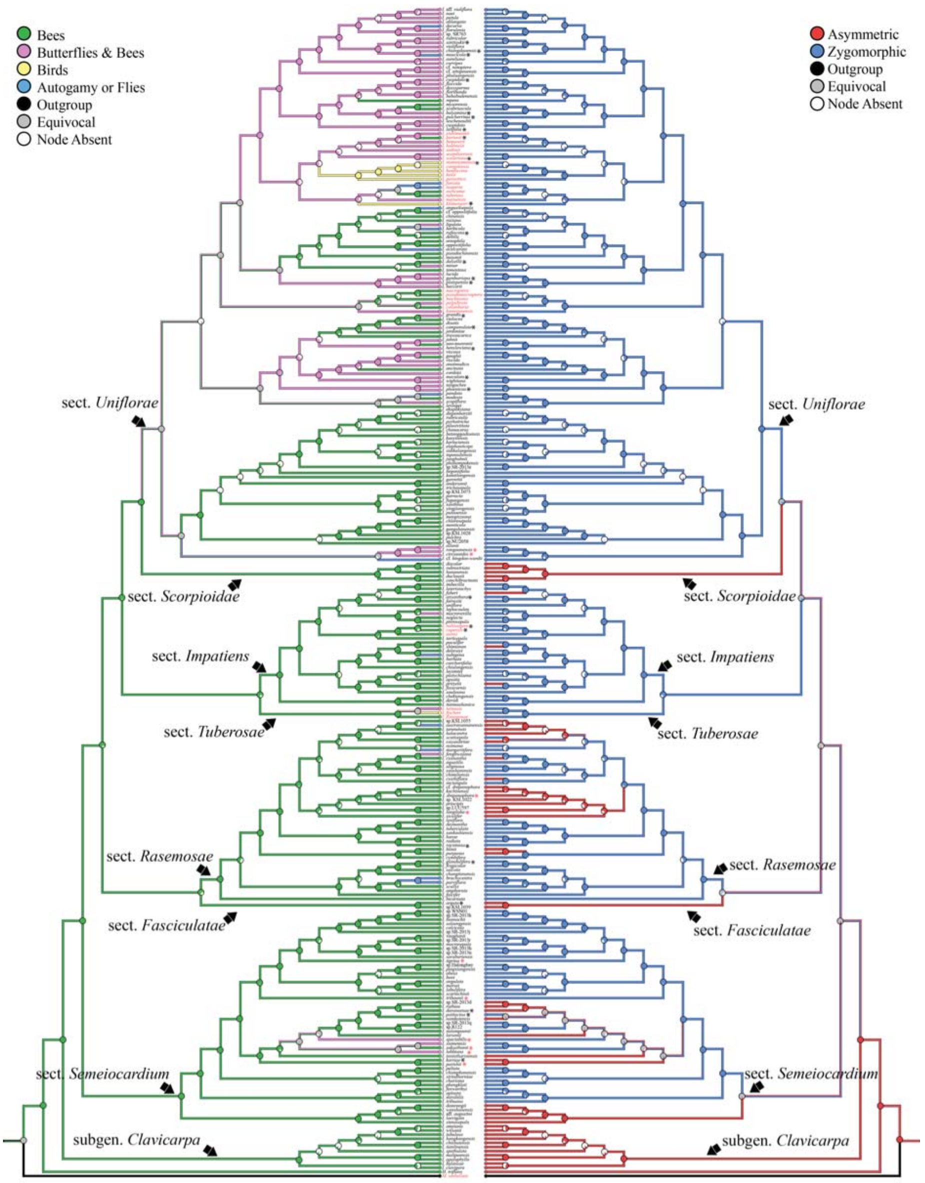

Fig. 7.S1. Parsimony Ancestral character state reconstruction of pollination systems (left) and corolla symmetry (right). Branch colors represent the most parsimonious ancestral character states reconstructed using the maximum clade credibility tree. Pie charts at nodes represent the proportion of BEAST trees for which a particular character state at a node was reconstructed, the node was absent, or reconstruction was equivocal, in case that ***H. triflora* was treated as asymmetrical corolla**. The species outside Asia were marked by red alphabets. The black asterisk (*) show the species with pollination studies in the table 1. The red Asterisk (*) shows the species with pollinator were observed by Saroj Ruchisansakun.

Table 7.2. Percentage of tree of each pollination state of ancestral node of Balsaminaceae and *Impatiens* from Ancestral state reconstruction.

Methods	Datasets	Balsaminaceae ancestral state	*Impatiens* ancestral state
		Bee: percentage (trees)	Bee percentage (trees)
Parsimony	Combined	**100** (9001)	**100** (9001)
	ITS	**100** (9001)	**100** (9001)
	atpB-rbcL	**100** (9001)	**100** (9001)
ML (Mk1)	Combined	**100** (9001)	**100** (9001)
	ITS	**100** (9001)	**100** (9001)
	atpB-rbcL	**100** (9001)	**100** (9001)

Table 7.3. Percentage of tree of each symmetry state of ancestral node of Balsaminaceae and *Impatiens* from Ancestral state reconstruction.

Hydrocera state	Datasets/ Methods	Balsaminaceae ancestral state: Percentage (trees)			*Impatiens* ancestral state: Percentage (trees)		
		Zygomo	Asymm	Equivo	Zygomo	Asymm	Equivo
Zygomorphic	Combined/ Parsimony	**93.56** (8421)	0.00 (0)	6.44 (580)	**93.56** (8421)	0.00 (0)	6.44 (580)
	Combined/ ML (Mk1)	**80.70** (7264)	0.00 (0)	19.30 (1737)	**91.06** (8196)	0.00 (0)	8.94 (805)
	atpB-rbcL/ Parsimony	**100.00** (9001)	0.00 (0)	0.00 (0)	**62.37** (5614)	1.06 (95)	36.57 (3292)
	atpB-rbcL/ ML (Mk1)	27.82 (2504)	0.00 (0)	**72.18** (6497)	34.17 (3076)	0.00 (0)	**65.83** (5925)
	ITS/ Parsimony	**100.00** (9001)	0.00 (0)	0.00 (0)	**100.00** (9001)	0.00 (0)	0.00 (0)
	ITS/ ML (Mk1)	**100.00** (9001)	0.00 (0)	0.00 (0)	**99.69** (8973)	0.00 (0)	0.31 (28)
Asymmetrical	Combined/ Parsimony	0.00 (0)	**100.00** **(9001)**	0.00 (0)	0.00 (0)	**100.00** **(9001)**	0.00 (0)
	Combined/ ML (Mk1)	0.00 (0)	0.00 (0)	**100.00** **(9001)**	0.06 (5)	0.00 (0)	**99.94** **(8996)**
	atpB-rbcL/ Parsimony	0.00 (0)	37.63 (3387)	**62.37** **(5614)**	0.00 (0)	37.63 (3387)	**62.37** **(5614)**
	atpB-rbcL/ ML (Mk1)	0.00 (0)	0.00 (0)	**100** **(9001)**	0.11 (10)	0.00 (0)	**99.89** **(8991)**
	ITS/ Parsimony	**82.68** **(7442)**	0.31 (28)	17.01 (1531)	**82.68** **(7442)**	0.31 (28)	17.01 (1531)
	ITS/ ML (Mk1)	**87.76** **(7899)**	0.00 (0)	12.24 (1102)	**97.93** **(8815)**	0.00 (0)	2.06 (186)

Table 7.4. Summarizing changes over trees in Pollination systems of *Impatiens*, a parsimony ancestral state reconstruction (unordered) using the trees from the Bayesian analysis.

Pollination systems shifts		Number of each kind across all mappings and trees from each DNA datasets: average (min-max)		
From	To	Combined	*atpB-rcbL*	ITS
Bees	Bimodal	**12.51** (4-21)	**13.36** (5-26)	**9.6** (4-20)
Bees	Birds	0.69 (0-3)	**1.48** (0-4)	0.76 (0-3)
Bees	Autogamy or flies	**8.29** (5-10)	**8.55** (3-12)	**8.25** (6-10)
Bimodal	Bees	**11.20** (4-20)	**12.29** (1-22)	**13.45** (4-19)
Bimodal	Birds	**2.26** (2-3)	**2.51** (0-4)	**2.19** (0-3)
Bimodals	Autogamy or flies	**3.68** (2-6)	**3.80** (2-7)	**3.74** (2-6)
Birds	Bees	0.02 (0-1)	0.23 (0-2)	0.09 (0-1)
Birds	Bimodal	0.38 (0-3)	0.35 (0-63)	0.37 (0-3)
Birds	Autogamy or flies	0.02 (0-1)	0.00 (0-1)	0.00 (0-1)
Autogamy or flies	Bees	0.47 (0-4)	0.73 (0-6)	0.19 (0-3)
Autogamy or flies	Bimodals	0.53 (0-3)	0.24 (0-4)	0.33 (0-2)
Autogamy or flies	Birds	0.00 (0-1)	0.02 (0-1)	0.00 (0-1)
Total shifts		40.05	43.56	38.97

Table 7.5. Summarizing changes over trees in corolla symmetry of *Impatiens*, Parsimony and Likelihood ancestral state reconstruction using the trees from BEAST analysis

Hydrocera floral symmetry state	DNA Datasets	Number of shifts of each kind across all mappings and trees: average (min-max)		
		Zygomorphic to Asymmetrical	Asymmetrical to Zygomorphic	Total shifts
Zygomorphic	Combine	16.79 (6-22)	3.30 (0-16)	20.74
	atpB-rbcL	16.62 (5-23)	4.63 (0-18)	21.25
	ITS	14.69 (10-17)	2.60 (0-8)	17.29
Asymmetric	Combine	13.21 (5-21)	6.73 (1-16)	20.13
	atpB-rbcL	15.10 (4-23)	6.77 (0-19)	21.87
	ITS	15.12 (8-18)	2.82 (0-11)	17.94

clade which comprises *I.* sect. *Uniflorae*, whereas some species are member of the smaller clade which comprises *I.* sect. *Tuberosae*. Madagascan species form a monophyletic clade and are placed in the big African clade. European and North American species form a monophyletic clade within the clade of *I.* sect. *Impatiens* (Fig. 7.1, 7.S1).

7.4.3. The evolution of pollination systems

The majority of the results are similar among the two separate gene trees and the combined data set (Table 7.2-7.5). In the sections below we therefore only mention the results from the combined dataset.

Bee pollination is ancestral in Balsaminaceae and *Impatiens* in both Parsimony and Maximum likelihood ancestral state reconstructions (Table 7.2 & Fig. 7.1). Then, it shifted to bimodal pollination, and to autogamy or fly pollination, multiple times (Table 7.4). The shift from bees to birds never occurred based on the combined and ITS analyses, but did exist in the analysis based on *atpB-rbcL* (Table 7.4). The reverse shift from bimodal to bee occurred relatively frequently. Bimodal pollination also shifted to autogamy or fly pollination and bird pollination multiple times (Table 7.4). The shifts between other pollination systems are less than 1 (Table 7.2). The average total number of shifts in pollination system is 40 shifts (ca. 14.23% of the possible shifts).

Shifts in pollination system appear phylogenetically concentrated in the clade that comprises sect. *Uniflorae* (Figs. 7.1 & 7.S1).

7.4.4. The evolution of corolla symmetry

The one-parameter Markov k-state model (Mk1) was selected over the AsymmMk model for shifts in corolla symmetry ($p=0.1915$ when *Hydrocera* was coded as zygomorphic: $p=0.8546$ when *Hydrocera* was coded as asymmetrical). The result of the ancestral state reconstruction of Balsaminaceae and *Impatiens* depends on the coding of *Hydrocera*. When *Hydrocera* was coded as zygomorphic, the ancestral state of Balsaminaceae and *Impatiens* was also zygomorphic, except for the results from the ML (Mk1) analysis of *atpB-rbcL*, which showed an equivocal result (Table 7.3). When *Hydrocera* was coded as asymmetrical, the ancestral state of Balsaminaceae and *Impatiens* was asymmetrical in the Parsimony analysis of the combined data but equivocal in the ML (Mk1) analysis of the combined analysis and the *atpB-rbcL* analysis , and zygomorphic in the Parsimony and ML (Mk1) analysis of ITS (Table 7.3).

Summarizing state change over trees shows a higher number of shifts from zygomorphic to asymmetrical than shifts from asymmetrical to zygomorphic (Table 7.5). The positions of corolla symmetry shifts on trees are scattered across several clades, regardless of the coding of *Hydrocera* (i.e. *I.* subgen. *Clavicarpa*, *I.* sect. *Scorpioidae*, *I.* sect. *Impatiens*, *I.* sect. *Racemosae*, *I.* sect. *Fasciculatae*, *I.* sect. *Semeiocardium*), comprising mostly Asian species (Fig. 7.1 & 7.S1).

7.5. DISCUSSION

Evolution of pollination systems in *Impatiens* is characterized by both pollination system shifts and divergent use of the same pollinator (cf. Stebbins, 1970). Bee pollination is the ancestral state for both Balsaminaceae and *Impatiens,* and the shifts to other pollination systems occurred multiple times. Furthermore, although it is unclear whether the ancestral *Impatiens* corolla was zygomorphic or asymmetrical, several shifts towards asymmetrical corollas have occurred, with some reversals. These shifts in corolla symmetry always occurred against a background of ancestral bee pollination. Shifts consistent with each of the two distinct pollinator-driven evolutionary processes are confined to particular clades.

The accuracy of our results from the analyses of pollinator-driven evolution depends on the correct inference of pollination systems, as the majority of sampled taxa has not been studied in the field. Although our inferences were based on over 54 individual pollination studies (Table 7.1), we acknowledge that our coding by functional pollinator group may lead to errors. In particular, pollination systems which are not represented among the studied species remain unknown and hence likely lead to underestimation of diversity in pollination systems and number of pollination system shifts. Given the strong associations between floral morphology and pollinator functional groups in *Impatiens* in particular (Abrahamczyk *et al*., 2017; Grey-Wilson, 1980; Ruchisansakun *et al*., 2016; Vogel, 1954), and flowering plants in certain regions in general (Johnson & Wester, 2017), our approach is useful for groups for which observations are lacking, and challenging due to a wide distribution range. However, most species in our analysis are from Asia (Fig. 7.1) where most pollination studies were done (Table 7.1). Hence we expect relatively few incorrect inferences. Furthermore, we included pollination studies that were performed on non-native species (e.g. *I. glandulifera*, which is native in the Hymalayan mountains and invasive in many European countries has been extensively studied in Europe) (Knuth, 1898;

Erpenbach, 2006; Nienhuis *et al*., 2009; Ugoletti *et al.,* 2013; Abrahamczyk *et al*., 2017). Although pollinators of invasive species are unlikely to have selected for large changes in floral syndrome traits over the relatively short time since the invasion, we still think that studies done on invasive species can reveal useful information on morphological fit and pollinator types (Abrahamczyk *et al*., 2017). This is particularly the case if pollinators in the native and invaded range of an *Impatiens* species represent similar functional pollinator groups, as is for instance the case with bumble bee species in Asia and Europe, which are both representatives of the genus *Bombus* (in fact, it is unlikely that a species with a relatively specialized floral morphology can invade a region without the presence of a functional pollinator niche, unless it is capable of autonomous self-pollination (Duffy & Johnson, 2017). The most problematic case with regards to our inference is the subtle difference between minute fly-pollinated species and autogamous species. Given the paucity of studies done for species with either pollination system, we have tentatively coded these similarly, although the only similarity may be in floral size, but not in other floral traits (Ruchisansakun *et al*. 2016; Abrahamczyk *et al*., 2017). Given that almost all shifts towards this floral syndrome occurred in isolated species, our categorisation does not affect the reconstructed number of shifts in the pollination system.

Impatiens is characterized by different pollination systems, such as bees, Lepidoptera and bees, birds, flies, and autogamous (Grey-Wilson, 1980; Ruchisansakun *et al*., 2016; Abrahamczyk *et al*., 2017). In our analysis, focusing on Asian species, bee pollination is the most common pollination system, followed by bimodal pollination by Lepidoptera and bees, and autogamy or fly pollination. Bird pollination is the least common and only found in Africa. The distribution of pollination systems among Asian species appears to be different from that in African species, which are mostly pollinated by either Lepidoptera or birds (Grey-Wilson, 1980; Janssens, 2008). This difference may to some extent reflect the different animal distributions between the areas. For example, bee genera such as *Bombus* and *Apis*, which are important pollinators of Asian *Impatiens* species, are uncommon in tropical Africa (Williams, 1998; Gupta, 2014). Birds, on the other hand, are an important pollination niche in tropical African plants, including many African *Impatiens* (Janecek *et al.,* 2015; Bartoš & Janecek, 2014, 2017). However, pollinator distributions per species cannot be the only explanation for the differences in pollination systems, as sunbirds are also present in Asia, whereas bird-pollination does not seem to occur. For instance, *I. phoenicea*, *I. coelotropis,* and *I. platyadena*, appear attractive to birds, but these species are pollinated by bees (Ramasubbu *et al.,* 2009; Ramasubbu *et al.,* 2011) or by bees and Lepidoptera (bimodal) (Sreekala *et al.,* 2008a; Sreekala *et al.,* 2008b). We propose that the different frequencies of pollination systems can be understood in a biogeographical context. Phylogenetic evidence suggests that Balsaminaceae originated and dispersed into Asia (Janssens *et al*., 2009). This may explain why bee-pollination, currently common among Asian species, was ancestral in this range. Colonization of tropical Africa, where a limited number of large bee species currently occurs, would then have to be associated with shifts to a new, locally available pollination niche (cf. Johnson, 1997; Van der Niet *et al*., 2014). Indeed, pollination system shifts occur mostly in the clade of *I.* sect. *Uniflorae* which comprises many African and Malagassy *Impatiens* species (Yuan *et al.,* 2004; Yu *et al.,* 2015).

The relatively small number of pollination system shifts among Asian *Impatiens* species, does not necessiarly signal the absence of pollinator-driven evolution. Indeed, shifts in floral symmetry between zygomorphic and asymmetrical corollas occurred several times in *Impatiens*. The polymorphic nature of *Hydrocera* in terms of corolla symmetry strongly affects our inference of the ancestral state of floral symmetry for both Balsaminaceae and *Impatiens*. This is presumably due to the phylogenetic position of *Hydrocera* as sister to the genus *Impatiens*. An in-depth intraspecific study is required to assess whether *Hydrocera* is ancestrally zygomorphic or asymmetrical. However, regardless of the ancestral state for the family and genus, shifts in floral symmetry have occurred around 20 times and appear to be reversible, although the number of shifts toward asymmetrical is higher than shifts toward zygomorphic in both cases (Table 7.5). Furthermore, shifts in floral symmetry always occur against a background of ancestral bee-pollination, only in Asian species (Figs. 7.1 & 7.S1). Based on the presumed function of asymmetrical corollas in terms of mediating precise different pollen placement, our result suggests several independent shifts in the use of the same pollinator (cf. Stebbins, 1970; Johnson, 2010; Armbruster, 2014). We probably underestimated the evolution of the divergent use of the same pollinator, because this may occur through other floral mechanisms than corolla asymmetry (Ruchisansakun *et al*., 2016). Interestingly, a similar mechanism of divergent use of the same pollinator appears to have evolved in African bird-pollinated *Impatiens* species, although the mechanism appears different (Bartoš & Janeček, 2014).

Shifts between pollination systems and floral symmetry can only partially explain the high diversity of *Impatiens*. In a recent meta-analysis of shifts in pollination systems, on average 25% of cladogenic events were associated with pollination system shifts (Van der Niet & Johnson, 2012). In our study, we found that ca. 14.2% of possible shifts were explained by shifts in different pollination systems. However, if divergent use is added to this, it rises to 21.6% of possible shift. Although we argue that we may have underestimated the extent of pollinator-driven evolution, it seems likely that other drivers of speciation may be at play in *Impatiens*. Many *Impatiens* species are habitat specialists and consequently have a narrow and fragmented distribution. Such isolation may drive allopatric speciation (Janssens, 2008). Moreover, Yuan *et al.* (2004) showed that *Impatiens* species vary widely in their chromosome number, and that this trait is evolutionarily labile. This may be indicative of fast chromosomal evolution, leading to reproductive isolation and the evolution of species diversity without appreciable divergence in floral traits (White, 1968). More research into the relatively importance of these different types of speciation is required to reconstruct and understand the evolution of *Impatiens*.

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