

# Parallel floral adaptations to pollination by fungus gnats within the genus *Mitella* (Saxifragaceae)

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## Abstract

The widespread pattern of parallel flower evolution as an adaptation for particular pollinator agents, known as “pollination syndromes”, has long drawn attention from evolutionary biologists. Here, we report parallel evolution of saucer-shaped flowers and an associated unusual pollination system within the lineage Heucherina, a group of saxifragaceous genera. Field observations reveal that 18 of 28 plant species studied are pollinated almost exclusively by fungus gnats (Mycetophilidae). Among the 18 species with a fungus-gnat pollination system, 13 have characteristic saucer-shaped flowers and are pollinated mainly by several unspecialized mycetophilid genera with short mouthparts. We performed phylogenetic analyses using nucleotide sequences of external and internal transcribed spacers of nuclear ribosomal DNA and reconstructed ancestral floral morphologies with an establishment of the model of floral character evolution under a maximum-likelihood framework. Our analysis indicates that there is significant directionality in the evolutionary shifts of floral forms in the Heucherina. The inferred phylogeny further supports four origins of saucer-shaped flowers, which is shared among 14 species that are traditionally classified into the genus *Mitella*. In addition, our analysis indicates the extensive polyphyly of genus *Mitella*, as also suggested previously. The results suggest that the flower-visiting fungus gnats have caused convergent selection for the saucer-shaped flower repeatedly evolved within Heucherina.

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## 1. Introduction

Among biological phenomena, parallel evolution is of great interest because it provides a foundation for studying ecological conditions that result in common adaptive phenotypes among independent populations in nature (Schluter et al. (2004)). Meanwhile, evolutionary parallelism has the potential to confound phylogenetic relationships of organisms through phenotypic convergence, thereby complicating interpretation of diversification in adaptive traits. Parallelism as adaptation for a particular set of pollinators is a widespread trend in flower evolution, often referred to

as pollination syndromes (Faegri and van der Pijl, 1979; Proctor et al., 1996; Johnson and Steiner, 2000; Fenster et al., 2004). Although there is still controversy about the prevalence of pollination syndromes amongst the perhaps 300,000 species of flowering plants (Waser et al., 1996; Hingston and McQuillan, 2000, but see Johnson and Steiner, 2000; Fenster et al., 2004; Pauw, 2006), it is inferred to be evidence for strong selection pressure toward particular suites of floral traits mediated by preference, behavior, and morphology of pollinators (Kevan and Baker, 1983; Schemske and Bradshaw, 1999; Bradshaw and Schemske, 2003; Stuurman et al., 2004). Recent advances in plant phylogenetics have accelerated our understandings about evolutionary dynamics of floral traits associated with specific pollinators (e.g., Johnson et al., 1998; Weller and Sakai, 1999; Goldblatt et al., 2001; Beardsley et al., 2003; Kay

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et al., 2005, and references therein). Nevertheless, most plant-pollinator relationships are still unexplored (Johnson and Steiner, 2000), thus there are numerous cases in which adaptive roles of floral traits have not been rigorously analyzed.

The riparian forest floor along small streams is a microhabitat of temperate regions with a characteristic herbaceous flora. The composition of the anthophilous insect fauna of this cool, shady, and damp environment is also characteristic, and is notably poor, e.g., in anthophiles such as bees (Mesler et al., 1980). Recently, Goldblatt et al. (2004) and Okuyama et al. (2004) independently discovered pollination by a previously neglected group of flower-visiting insects, namely fungus gnats (Mycetophilidae), in several species of Heucherina (a well-supported clade of Saxifragaceae comprising several genera, not corresponding to any Linnean rank but equivalent to Soltis et al.'s (1991) "Heuchera group"; see Soltis et al., 2001) in streamside microhabitats of Pacific Northwestern America and of Japan. Because these studies documented high effectiveness of this unusual pollination mode, we hypothesized a widespread presence of similar pollination systems in the Heucherina, especially because many Heucherina species in the riparian microhabitat have floral traits similar to those pollinated by fungus gnats, such as saucer-shaped, greenish flowers with linear, branched petals. If correct, it may suggest that parallel floral adaptation to pollination by fungus gnats may have occurred within Heucherina.

Here we provide evidence for fairly widespread convergent evolution of saucer-shaped flowers that are apparently adapted for pollination by fungus gnats within the genus *Mitella*, a polyphyletic member of the Heucherina. The Heucherina is potentially an ideal system for studying the dynamics of floral evolution because it has quite diverse floral phenotypes and corresponding pollination systems, and limited geographic distribution mostly confined to North America and Japan, which facilitates extensive surveys on the life history traits of the whole lineage. We utilize comprehensive data on their phylogenetic relationships, floral morphology, and pollination systems to analyze patterns of floral trait diversification in Heucherina, and to explore the ecological backgrounds existing behind parallelism. We further discuss the possibility that the traditional treatment of polyphyletic *Mitella* as a single genus has been partially caused by parallel adaptation to pollination by fungus gnats, which appears to be a potential, unrecognized floral syndrome in very moist microhabitats in temperate forests.

## 2. Materials and methods

### 2.1. Study species

Heucherina is a monophyletic group of perennials containing nine genera and about 80 species (Soltis et al., 2001). Among these species, we sampled 53 species representing all nine genera as ingroups for the analyses of

ancient shifts in floral morphology (Table 1). We were unable to obtain samples of *Mitella diversifolia* (a close relative of *M. stauropetala* and *M. trifida*, Soltis and Kuzoff, 1995; Wakabayashi, 2001), *Lithophragma maximum*, *L. trifoliatum*, and several species of genus *Heuchera*. However, further addition of these species is unlikely to alter the results of our analyses, as the omitted species always have floral morphology similar to those represented in our samples, and our sampling covers all major lineages of *Heuchera*, *Lithophragma*, and *Mitella*. For convenience, eastern and western populations of *M. japonica* as well as some varieties within *M. furusei* and *M. stylosa* were treated as distinct species, as they are genetically and/or morphologically distinct to each other (Okuyama et al., 2005; Okuyama unpublished data). We used *Darmera peltata*, *Rodgersia podophylla*, and *Peltoboykinia tellimoides* as outgroups.

### 2.2. Collection of DNA sequence data and phylogenetic analysis

Most DNA sequence data of nuclear ribosomal ETS and ITS from the North American and Asian species were generated using standard methods described previously (Okuyama et al., 2005), whereas some sequence data (ITS of *Lithophragma affine* from Kuzoff et al., 1999 and several Asian species from Okuyama et al., 2005) were obtained from previously published sequences (for the localities of plant collections and the DDBJ/NCBI accession numbers and original references, see Table 1). We did not include chloroplast gene sequences within our analyses because we consider them as inappropriate for inferring ancestral states, as chloroplast genes are more prone to introgress than nuclear ribosomal genes via rare interspecific hybridization events in Heucherina (Soltis and Kuzoff, 1995; Okuyama et al., 2005). Alignment was conducted using Sequence Navigator (Perkin-Elmer, Foster City, CA) and obvious errors were corrected manually. Gaps were unambiguously coded as separate characters by using the methods of Simmons and Ochoterena (2000). Maximum parsimony (MP) trees were constructed using PAUP\* 4.0 (Swofford, 2003) with heuristic search (tree-bisection-reconnection [TBR] algorithm for branch swapping was used) saving all optimal tree topologies (MulTrees) in each of the 100 random sequence addition replicates. To assess topological uncertainty, bootstrapping (10,000 replicates) was also performed, using TBR branch swapping and ten random sequence addition replicates with saving a maximum of 100 trees in each replicate.

To obtain the posterior distribution of phylogenetic trees from our data set, Bayesian phylogenetic inference was also conducted using BayesPhylogenies (Pagel and Meade, 2004; available from [www.evolution.rdg.ac.uk](http://www.evolution.rdg.ac.uk)). We used GTR+G base substitution model for all nucleotide characters. In addition, we used the mlp model for binary gap characters. Multistate gap characters were excluded from the data set for Bayesian analysis, as they

Table 1  
The collection localities, voucher information, and DDBJ/Genbank accession numbers of the *Heucherina* plant used for the present phylogenetic analyses

Species	Location	Voucher	ETS	ITS
<i>Heucherina</i>				
<i>Bensoniella oregona</i> (Abrams & Bacig.) Morton	Bear Camp, Curry Co., OR, USA	Leads s.n. (WS: WSU77821)	AB291995	AB292019
<i>Conimitella williamsii</i> (D.C. Eat.) Rydb.	Little Belt Mts., Lewis & Clark Co., MT, USA	Lesica 8603 (NY)	AB291996	AB292020
<i>Elmera racemosa</i> (Wats.) Rydb.	Early Winters Creek, Okanogan Co., WA, USA	Okuyama 047092 (KYO)	AB248766	AB248849
<i>Heuchera americana</i> L.	Pennel Run, Delaware St. For., PA, USA	Cultivated in Kyoto University	AB291997	AB292021
<i>H. chlorantha</i> Piper	Haida Pt., Queen Charlotte Islands, BC, Canada	Caldes & Taylor 35399 (WS: WSU263728)	AB291998	AB292022
<i>H. cylindrica</i> Dougl. ex Hook.	St. Joe Baldy, Benewah Co., ID, USA	Okuyama 04626HC1 (KYO)	AB248767	AB248850
<i>H. elegans</i> Abrams	San Gabriel Mts., Los Angeles Co., CA, USA	Bornstein, Collins, & O'Brien s.n., Cultivated in UCBG (No. 2001.0668)	AB291999	AB292023
<i>H. glabra</i> Willd. ex Roemer & J.A. Schultes	Chinook Pass, Yakima Co., WA, USA	Okuyama 047071 (KYO)	AB248768	AB248851
<i>H. grossulariifolia</i> Rydb.	Riggins, Idaho Co., ID, USA	Cultivated in University of Idaho	AB248769	AB248852
<i>H. maxima</i> Greene	Santa Cruz Island, Santa Barbara Co., CA, USA	Bartholomew & Zadnik 715, Cultivated in UCBG (No. 80.0100)	AB292000	AB292024
<i>H. merriamii</i> Eastw.	Kangaroo Ridge, Siskiyou Co., CA, USA	Raiche 10104, Cultivated in UCBG (No. 81.0692)	AB292001	AB292025
<i>H. micrantha</i> Dougl. ex Lindl.	Diablo, Whatcom Co., WA, USA	Cultivated in Kyoto University	AB248770	AB248853
<i>H. parvifolia</i> Nutt. ex Torr. & Gray	Aguirre Springs Rec. Area, Dona Ana Co., NM, USA	Spellenberg & Sweeney 13525 (NY)	AB292002	AB292035
<i>H. pilosissima</i> Fisch. & C.A. Mey.	Landels-Hill Big Creek Reserve, Monterey Co., CA, USA	Cultivated in Kyoto University	AB292003	AB292036
<i>H. richardsonii</i> R. Br.	Adams Co., IL, USA	Tyson s.n., Cultivated in UCBG (No. 86.0106)	AB292004	AB292026
<i>H. rubescens</i> Torr.	Sandia Creek, Sandia Mountain, Bernalillo Co., NM, USA	Fishbein 4600 (WS: WSU366880)	AB292005	AB292027
<i>H. villosa</i> Michx.	Whiteside Mountain, Nantahala Nat. For., Jackson Co., NC, USA	Cultivated in UCBG (No. 86.0656)	AB292006	AB292028
<i>Lithophragma affine</i> Gray	Humboldt Co., CA, USA	Bohm 1671 (WS: WSU343725)	AB292007	AF158921 <sup>a</sup>
<i>L. bolanderi</i> Gray	Sally Keys Lake, Fresno Co., CA, USA	Kruckeberg s.n. (WS: WSU236030)	AB292008	AB292029
<i>L. campanulatum</i> T.J. Howell	Yreka, Siskiyou Co., CA, USA	Bjork 5329 (WS: WSU362787)	AB292009	AB292030
<i>L. cymbalaria</i> Torr. & Gray	La Panza Rd., San Luis Obispo County, CA, USA	Schultz s.n. (WS: WSU317786)	AB292010	AB292031
<i>L. glabrum</i> Nutt.	Lower Granite Dam, Garfield Co., WA, USA	Soltis 2065 (WS: WSU363397)	AB292011	AB292032
<i>L. heterophyllum</i> (Hook. & Arn.) Torr. & Gray	Ukiah, Mendocino Co., CA, USA	Soltis & Soltis 2091 (WS: WSU342070)	AB292012	AB292033
<i>L. parviflorum</i> (Hook.) Nutt. ex Torr. & Gray	Mary Minerva McCloskey Memorial St. Pk. ID, USA	Okuyama & Pellmyr 046181 (KYO)	AB248771	AB248854
<i>L. tenellum</i> Nutt.	Fox Range, Washoe Co., NV, USA (NYBG)	Tiehm 14172 (NY)	AB292013	AB292034
<i>Mitella acerina</i> Makino	Ashu, Miyama, Kyoto Pref., Japan	Okuyama 039025A (KYO)	AB163594 <sup>b</sup>	AB163657, <sup>b</sup> AB163720 <sup>b</sup>
<i>M. brewerii</i> Gray	St Joe Baldy, Benewah Co., ID, USA	Okuyama 046261B (KYO)	AB248775	AB248858
<i>M. caulescens</i> Nutt.	Laird Park, Latah Co., ID, USA	Okuyama 047031 (KYO)	AB248776	AB248859
<i>M. diphylla</i> L.	Mississippi Palisades, Carrol Co., Illinois, USA	Hess s.n. (KYO)	AB248777	AB248860
<i>M. doiana</i> Ohwi	Yaku-Shima, Kagoshima Pref., Japan	Okuyama 035161 (KYO)	AB163560 <sup>b</sup>	AB163623, <sup>b</sup> AB163686 <sup>b</sup>
<i>M. formosana</i> (Hayata) Masamune	Nantou Hsien, Jenai Hsiang, Mei Feng, Taiwan	Ohwi-TW047 (MAK)	AB163559 <sup>b</sup>	AB163622, <sup>b</sup> AB163685 <sup>b</sup>
<i>M. furusei</i> Ohwi var. <i>furusei</i>	Misakubo, Shizuoka Pref., Japan	Okuyama 035011 (KYO)	AB163588 <sup>b</sup>	AB163651, <sup>b</sup> AB163714 <sup>b</sup>
<i>M. furusei</i> Ohwi var. <i>subramosa</i> Wakab.	Kusukawa, Nachi-Katsura, Wakayama Pref., Japan	Okuyama 039175 (KYO)	AB163601 <sup>b</sup>	AB163664, <sup>b</sup> AB163727 <sup>b</sup>
<i>M. integripetala</i> H. Boissieu	Mt. Minami-Shokan-take, Uryu, Hokkaido Pref., Japan	Okuyama 0390829 (KYO)	AB163547 <sup>b</sup>	AB163610, <sup>b</sup> AB163673 <sup>b</sup>
<i>M. japonica</i> Maxim. eastern strain	Akame 48 Falls, Nabari, Mie Pref., Japan	Cultivated in Kyoto University	AB292016	AB292039

Table 1 (continued)

Species	Location	Voucher	ETS	ITS
<i>M. japonica</i> Maxim. western strain	Amano-Iwato, Takachiho, Miyazaki Pref., Japan	Cultivated in Kyoto University	AB248782	AB248865
<i>M. kiusiana</i> Makino	Takachiho-Kyo, Takachiho, Miyazaki Pref., Japan	MAK 320814 (MAK)	AB163567 <sup>b</sup>	AB163630, <sup>b</sup> AB163693 <sup>b</sup>
<i>M. koshiensis</i> Ohwi	Miyazaki, Asahi, Toyama Pref., Japan	Cultivated in Makino herbarium, Tokyo Metropolitan University	AB163587 <sup>b</sup>	AB163650, <sup>b</sup> AB163713 <sup>b</sup>
<i>M. nuda</i> L.	Mt. Kitami-Fuji, Rubeshibe, Hokkaido Pref., Japan	MAK 331775 (MAK)	AB163549 <sup>b</sup>	AB163612, <sup>b</sup> AB163675 <sup>b</sup>
<i>M. ovalis</i> Greene	Fletcher Canyon, Olympic Nat. For., Jefferson Co., WA, USA	Okuyama 047099 (KYO)	AB248778	AB248861
<i>M. pauciflora</i> Rosend.	Tochimoto, Kiso-Fukushima, Nagano Pref., Japan	Okuyama 035271 (KYO)	AB163572 <sup>b</sup>	AB163635, <sup>b</sup> AB163698 <sup>b</sup>
<i>M. pentandra</i> Hook.	St. Joe Baldy, Benewah Co., USA	Okuyama 047011 (KYO)	AB248779	AB248862
<i>M. stauropetala</i> Piper.	W Palouse Range, Latah Co., ID, USA	Okuyama 046201 (KYO)	AB248780	AB248863
<i>M. stylosa</i> H. Boissieu var. <i>makinoi</i> (H. Hara) Wakab.	Omiya-dani, Ikeda, Tokushima Pref., Japan	Okuyama 034231 (KYO)	AN163563 <sup>b</sup>	AB163626, <sup>b</sup> AB163689 <sup>b</sup>
<i>M. stylosa</i> H. Boissieu var. <i>stylosa</i>	Seiho-ji, Fujiwara, Mie Pref., Japan	Cultivated in Makino herbarium, Tokyo Metropolitan University	AB163566 <sup>b</sup>	AB163629, <sup>b</sup> AB163692 <sup>b</sup>
<i>M. trifida</i> Graham	Mt. Aix, Yakima Co., WA, USA	Okuyama 047062 (KYO)	AB248781	AB248864
<i>M. yoshinagae</i> H. Hara	Kisawa, Tokushima Pref., Japan	Cultivated in Kyoto University	AB292017	AB292040
<i>Tellima grandiflora</i> (Pursh) Dougl. ex Lindl.	Grandy Lake, Skagit Co., WA, USA	Okuyama 0470913 (KYO)	AB248772	AB248855
<i>Tiarella cordifolia</i> L.	Perkins Clearing, Adirondack Pk., Hamilton Co., NY, USA	Okuyama 067011 (KYO)	AB292014	AB292037
<i>T. polyphylla</i> D. Don	Mt. Kitami-fuji, Rubeshibe, Hokkaido Pref., Japan	Okuyama 026232 (KYO)	AB163545 <sup>b</sup>	AB163608, <sup>b</sup> AB163671 <sup>b</sup>
<i>T. trifoliata</i> L. var. <i>trifoliata</i>	Federation Forest St. Pk., King Co., WA, USA	Okuyama 047061 (KYO)	AB248773	AB248856
<i>T. trifoliata</i> L. var. <i>unifoliata</i> (Hook.) Kurtz	Laird Park, Latah Co., ID, USA	Okuyama 047034 (KYO)	AB292015	AB292038
<i>Tohmiea menziesii</i> (Pursh) Torr. & Gray	Fletcher Canyon, Olympic Nat. For., Jefferson Co., WA, USA	Okuyama 047097 (KYO)	AB248774	AB248857
Outgroups				
<i>Darmera peltata</i> (Torr. ex Benth.) Voss	Mt. Timpanogos, Utah Co., UT, USA	Voucher No. missing (NY)	AB292018	AB292041
<i>Peltoboykinia tellimoides</i> (Maxim.) Hara	Tochimoto, Kiso-Fukushima, Nagano Pref. Japan	Okuyama 035251 (KYO)	AB248764	AB248847
<i>Rodgersia podophylla</i> A. Gray	Chokei-daira, Fukaura, Aomori Pref. Japan	Okuyama 039101 (KYO)	AB248765	AB248848

Note: Sources of specimens are given in parentheses (NY, Steere Herbarium of New York Botanical Garden; WS, Marion Ownbey Herbarium of Washington State University; UCBG, University of California-Berkeley Botanical Garden; KYO, Kyoto University Museum; MAK, Makino Herbarium, Tokyo Metropolitan University).

<sup>a</sup> Sequences from Kuzoff et al. (1999).

<sup>b</sup> Sequences from Okuyama et al. (2005).

are prohibited by the software. Two independent runs of Markov chain Monte Carlo (MCMC) simulation were allowed to go for three million generations each, with trees sampled every 5000 generations to achieve independence among samples. The likelihood scores of obtained trees were plotted to confirm that they reached stationarity well before the first 101 trees of each run, which were discarded as burn-in. As a result, 1000 trees were retained and used for subsequent analyses. In addition, a majority-rule consensus tree (hereafter referred to as Bayesian consensus tree) was constructed using the 1000 sampled trees, and branch lengths were then recalculated using PAUP\* 4.0 with the phylogenetic criterion set to likelihood, with optimal base substitution model, GTR+I+G, and model parameters selected by Akaike's information criterion

(AIC) using Modeltest (Posada and Crandall, 1998). Polytomies were randomly broken up to obtain a fully resolved tree, and branches estimated to have zero length were arbitrarily assigned the minimum length (0.00001), as the subsequent analyses of ancestral character reconstruction do not accept zero-branch length. The outgroups were excluded from all the trees obtained for the subsequent analyses, as they were only used for rooting the phylogenetic trees.

### 2.3. Reconstruction of ancestral floral morphologies

Maximum-likelihood estimation of ancestral floral traits was performed using BayesTraits (Pagel, 1994, 1999; Pagel et al., 2004; available from [www.evolution.rdg.ac.uk](http://www.evolution.rdg.ac.uk)) by



running the Maximum-Likelihood analysis method with 100 iterations per tree. Floral morphologies of the ingroup species were classified into the following three categories (see Fig. 1): flowers characterized by strongly elongated and projected styles and stamens (“projected”), flowers characterized by erect calyx, and anthers and stigmas located well below the tips of the calyx lobes (“enclosed”), and flowers characterized by flattened and saucer-shaped hypanthium with very short styles and stamens (“saucer-shaped”). This categorization scheme was chosen for reconstruction of ancestral floral morphologies because the difference among the categories is large and clear and involves major differences in steric arrangement of calyx lobes, anther, stigma, and nectary, thus likely reflecting divergent pollination systems. The character coding applied was based on several taxonomic sources (Hitchcock and Cronquist, 1961; Taylor, 1965; Elvander, 1993) and/or by directly checking herbarium specimens.

Best-fit models for character transition rate parameters were selected by likelihood-ratio test ( $\alpha = 0.05$ ) for each of 1000 MCMC samples of trees, starting from the six-parameter, most complex model to simpler models with stepwise approach. When we found a significant decrease in the likelihood score in part of the trees tested, we considered it a signal for rejection of that parameter restriction. After a model for character evolution was built, the relative likelihoods of alternative state for 17 focal nodes of the Bayesian consensus tree were determined with “local estimators” (Pagel, 1999) using the “fossil” command. Significant support for one ancestral state over another at each node was recognized when difference in the likelihoods exceeded conventional cutoff point of two log units. Because our study suggested multiple ori-

gins of the saucer-shaped floral morphology, we further assessed phylogenetic uncertainty of our estimate by the most recent common ancestor (MRCA) approach (Pagel et al., 2004), using the 1000 MCMC samples of trees. The relative likelihoods of alternative floral traits at the MRCA of all the species having saucer-shaped flowers were compared in each of the 1000 trees by the “fossil” command.

#### 2.4. Determining pollination systems

We determined the pollination systems of 28 ingroup species using direct field observations and supplementing them by published records (Table 2). Direct observations were made over a period of 6 years (2002–2007) at 32 study sites in Japan and North America for 23 species. Specifically, as we aimed to clarify the pollination systems of plant species bearing saucer-shaped flowers, which is characteristic of the genus *Mitella*, our observation covered 13 of 14 species of *Heucherina* having saucer-shaped flowers. We spent 12–48 h observing flower visitation by insects in each species in such a way that it covered all hours of daylight (Table 2). Moreover, as we documented highly specific pollination systems mediated by single species of the fungus-gnat genus *Gnoriste* in several species, ecological stability of such specialized systems was examined based on our field observations over 5 years and previous reports (Goldblatt et al., 2004; Okuyama et al., 2004; see Table 2). During direct observation, we captured all insects that visited flowers (after visit completion) and examined pollen load and estimated their visitation frequency from the number of insects collected to determine principal pollinators of the focal plants. For convenience, flower visitors with at least 10 pollen grains on their bodies were regarded as potential pollinators (Table 3). This number was chosen because most of the flower visitors (potentially effective pollinators) of *Heucherina* had much more pollen (usually >100, see Okuyama et al., 2004) on their body, and thus those with fewer than 10 pollen grains (33% of total flower visitors collected) would not contribute significantly to pollination, given that ovule numbers per flower of *Mitella* plants almost always exceed 40 and reaches as high as 130. Our sample of 140 flower visitors with fewer than 10 pollen grains each consisted of Mycetophilidae (38%), Empididae (36%), Syrphidae (12%), and other minute insects (14%). Note that the visitation frequency of Mycetophilidae was fairly underestimated in our study, because they were the most active amongst the flower visitors collected on most of *Heucherina* species and in most cases one insect visits flowers on multiple inflorescences (Okuyama et al., 2004, and Y.O., personal observations). Therefore, exclusion of the insects with fewer than 10 pollen grains does not affect the overall conclusion that the fungus gnats are acting as the principal pollinators of 18 of the *Heucherina* species studied.

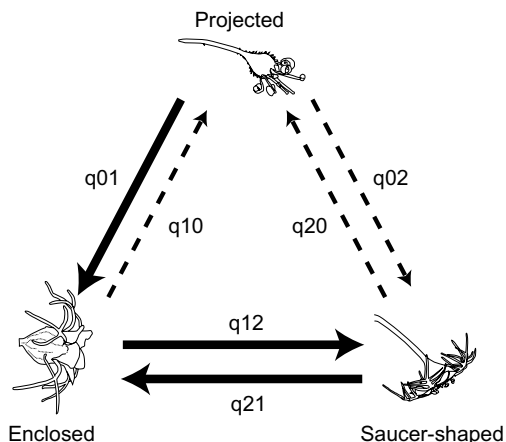


Fig. 1. Schematic diagram of character transitions among three categories of floral morphologies observed in *Heucherina*. There are a maximum of six parameters in the model of character evolution, with each parameter ( $q_{01}$ ,  $q_{02}$ ,  $q_{10}$ ,  $q_{12}$ ,  $q_{20}$ ,  $q_{21}$ ) corresponding to the probability of the character transition. The arrows in bold represent the three parameters that were shown to be greater than the remaining three, which are represented as dashed arrows.

Table 2  
Study site locations, study dates and periods, and references used for determining the pollination systems of the *Heuchera*

Species	Location <sup>a</sup>	Study date in the present study (observation period)	Reference used for determining pollination systems
<i>Heuchera cylindrica</i>	St. Joe Baldy, Benewah Co., ID, USA (SJ)	30 Jun. 04 (12 h)	Present study
<i>H. grossulariifolia</i>	—	—	Segraves and Thompson (1999), Nuismer and Cunningham (2005)
<i>H. micrantha</i>	Diablo, WA, USA	21 Jun. 05 (12 h)	Present study
<i>Lithophragma parviflorum</i>	—	—	Thompson and Pellmyr (1992)
<i>Mitella acerina</i>	Ashu, Miyama, Kyoto Pref, Japan	30 Apr. 04 (12 h)	Present study, Okuyama et al. (2004)
<i>M. breweri</i>	St Joe Baldy, Benewah Co., ID, USA (SJ)	26, 30 Jun. 04 (16 h)	Present study
	Rainy Lake, Wenatchee Nat. For., Chelan Co., WA, USA (RL)	21 Jun. 05 (2 h)	
<i>M. caulescens</i>	Laird Park, Latah Co., ID, USA	19–22 Jun. 04, 11,13,14 Jun. 05 (30 h)	Present study
<i>M. diphylla</i>	—	—	Graenicher (1909)
<i>M. furusei</i> var. <i>furusei</i>	Misakubo, Shizuoka Pref., Japan (MS)	1 May 03 (6 h)	Present study
	Fujinami-Dani, Sakauchi, Gifu Pref. (FN)	2 May 05 (12 h)	
<i>M. furusei</i> var. <i>subramosa</i>	Kibune-Okuno-In, Kyoto, Kyoto Pref., Japan (K)	29 Apr. 04 (4 h)	Present study, Okuyama et al. (2004)
	Mt. Daimonji, Kyoto, Kyoto Pref., Japan (DA)	18 Apr. 04 (6 h)	
	Ashu, Miyama, Kyoto Pref, Japan (AS)	30 Apr. 04 (12 h)	
	Akame 48 Falls, Nabari, Mie Pref., Japan (A)	27 Apr. 03, 26 Apr. 06 (6 h)	
	Mt. Tara, Isahaya, Nagasaki Pref. Japan (TR)	18, 19 Apr. 06 (6 h)	
<i>M. integripetala</i>	Mt. Minami-Shokan-take, Uryu, Hokkaido Pref., Japan	25 Jun. 03 (12 h)	Present study, Okuyama et al. (2004)
<i>M. japonica</i> Eastern strain	Akame 48 Falls, Nabari, Mie Pref., Japan	27 Apr. 03, 14 Apr. 05 (16 h)	Present study
<i>M. japonica</i> Western strain	Amano-Iwato, Takachiho, Miyazaki Pref., Japan (AM)	10, 11 Apr. 04 (12 h)	Present study
	Gokanosho, Izumi, Kumamoto Pref., Japan (GI)	20 Apr. 05 (4 h)	
<i>M. kiusiana</i>	Takachiho-Kyo, Takachiho, Miyazaki Pref., Japan (T)	7 Apr. 04 (6 h)	Present study
	Otaki, Itsuki, Kumamoto Pref., Japan (I)	9 Apr. 04, 21 Apr. 05 (12 h)	
<i>M. koshiensis</i>	Mikawa, Nigata Pref., Japan	7, 8 May 04 (16 h)	Present study
<i>M. nuda</i>	Mt. Kitami-Fuji, Rubeshibe, Hokkaido Pref., Japan (RB)	20 Jun. 03 (12 h)	Present study
	Kanoko-zawa, Oketo, Hokkaido Pref., Japan (OK)	10, 11 Apr. 07 (12 h)	
<i>M. ovalis</i>	Lake Sylvia St. Pk., Grays Harbor Co., WA, USA	7 Jul. 04 (4 h)	Present study
	Pete's Creek, Olympic Nat. For., Grays Harbor Co., WA, USA	19 Jun. 05 (8 h)	
<i>M. pauciflora</i>	Mt. Hakusan, Ishikawa Pref., Japan (HS)	21 May 03 (6 h)	Present study
	Ashu, Miyama, Kyoto Pref., Japan (AS)	10 Apr. 03 (6 h)	
	Kibune-Okuno-In, Kyoto, Kyoto Pref., Japan (K)	18 Apr. 03, 14 Apr. 05 (12 h)	
	Nishi-Iyayama, Tokushima Pref., Japan (NI)	21 Apr. 03 (2 h)	
	Iya, Ikeda, Tokushima Pref., Japan (IK)	23 Apr. 03 (8 h)	
	Oda-Miyama, Oda, Ehime Pref., Japan (OD)	16 Apr. 04 (8 h)	
	Akame 48 falls, Mie Pref., Japan (A)	14 Apr. 05 (6 h)	
<i>M. pentandra</i>	St. Joe Baldy, Benewah Co., ID, USA (SJ)	26,30 Jun. 04 (16 h)	Present study
	Rainy Lake, Wenatchee Nat. For., Chelan Co., WA, USA (RL)	21 Jun. 05 (2 h)	
<i>M. stauropetala</i>	W Palouse Range, Latah Co., ID, USA	20,23 Jun. 04 (12 h)	Present study; Pellmyr et al. (1996)
<i>M. stylosa</i> var. <i>makinoi</i>	Irazu Gulch, Higashi-tsuno, Kochi Pref., Japan	14, 15 Apr. 04 (12 h)	Present study, Okuyama et al. (2004)
<i>M. stylosa</i> var. <i>stylosa</i>	Fujiwara, Mie Pref., Japan	29 Apr. 03, 30 Apr. 05 (16 h)	Present study

(continued on next page)

Table 2 (continued)

Species	Location <sup>a</sup>	Study date in the present study (observation period)	Reference used for determining pollination systems
<i>M. yoshinagae</i>	Otaki, Itsuki, Kumamoto Pref., Japan (I) Horikiri-Dani, Taragi, Kumamoto Pref., Japan (HK)	9 Apr. 04 (4 h) 19 Apr. 05 (8 h)	Present study
<i>Tellima grandiflorum</i>	—	—	Weiblen and Brehm (1996)
<i>Tiarella cordifolia</i>	—	—	Motten (1986)
<i>T. polyphylla</i>	Kuroguchi Trail, Hase, Nagano Pref. Japan (KG) Murii Trail, Maruseppu, Hokkaido Pref., Japan (MR) Kanoko-zawa, Oketo, Hokkaido Pref., Japan (OK)	9 Jun. 03 (8 h) 22 Jun. 03 (8 h) 11 Jun. 07 (2 h)	Present study
<i>T. trifoliata</i> var. <i>unifoliata</i>	W Palouse Range, Latah Co., ID, USA	20, 23 Jun. 04 (12 h)	Present study
<i>Tolmiea menziesii</i>	Grandy Lake, Mt. Baker Snoqualmie Nat. For., Skagit Co., WA, USA (GL) Pete's Creek, Olympic Nat. For., Grays Harbor Co., WA, USA (PC)	9 Jul. 04 (4 h) 19 Jun. 05 (8 h)	Present study, Weiblen and Brehm (1996), Goldblatt et al. (2004)

<sup>a</sup> The letters in parentheses are the abbreviations of study sites used in Table 3.

### 3. Results

#### 3.1. Phylogeny of *Heucherina* inferred from nuclear ribosomal DNA sequences

Number of sites in the aligned data matrix of ETS, ITS-1, 5.8S, ITS-2, and indels were 502, 284, 164, 253, and 71, respectively. The parsimony analysis of nuclear ribosomal external and internal spacer regions (ETS and ITS) of the entire *Heucherina* resulted in 419 maximum-parsimony (MP) trees (1257 steps, CI = 0.6516, RI = 0.7947). A Bayesian phylogenetic analysis supported 92% of the nodes that were retained in the strict consensus of the 419 MP trees with high posterior probability (>0.90), and the remaining nodes with moderate posterior probability (0.60–0.85). *Lithophragma* and *Tiarella* were each recovered as a monophyletic group with very strong support (99–100% bootstrap and 1.00 posterior probability). In contrast, *Heuchera* and *Mitella* were not supported as monophyletic. Notably, the genus *Mitella* was separated into several unrelated clades, each of which corresponded to a morphologically defined section (Fig. 2). At the supra-generic level, several well-supported clades that have never been recovered consistently in previous studies were observed. For example, *Heucherina* was separated into two major clades, A and B, where the former consists of *Bensoniella*, *Heuchera*, *Lithophragma*, *Tolmiea*, and several species of *Mitella* (clade A), and the latter consists of *Conimitella*, *Elmera*, *Tiarella*, and the remaining *Mitella* species (clade B). Moreover, the internal clades within clade B were generally well supported (>87% bootstrap and >0.98 posterior probability). Overall, although inter- and intrageneric introgression especially in chloroplast genome is suggested to have occurred in *Heucherina* (e.g., Soltis and Kuzoff, 1995), apparent introgression patterns (i.e., sharing genotype across distantly related species, see

Soltis et al., 1991) or other anomalous topologies were not observed in our data set, implying that the results of the phylogenetic analyses were reliable.

#### 3.2. Parallelism of saucer-shaped flowers in *Heucherina*

The inferred phylogeny, coupled with information on floral traits, enabled us to build the model of floral character evolution of *Heucherina*, with 53 ingroup species being categorized unambiguously into three groups that have projected, enclosed, and saucer-shaped flowers, respectively. A likelihood-ratio test ( $\alpha = 0.05$ ) resulted in a selection of best-fit model as two-parameter model with parameter restrictions of  $q_{01} = q_{12} = q_{21}$ , and  $q_{02} = q_{10} = q_{20}$  for all the 1000 Bayesian trees and the Bayesian consensus tree. The selected model resulted in the best log-likelihood scores among the 41 possible, two-parameter models, with an average of  $43.80 \pm 1.56$  ( $\pm$ sd), and the score was only 0.53 worse on average than the most complex, six-parameter model ( $P > 0.95$ , likelihood-ratio test). A rate parameter  $q_{01} = q_{12} = q_{21}$ , with an average of  $36.76 \pm 3.72$ , was always significantly larger than the other rate parameter  $q_{02} = q_{10} = q_{20}$  with an average of  $0.83 \pm 1.73$ , indicating that there were clear evolutionary trends such that the floral morphology changed more easily from projected to enclosed morphs or between enclosed and saucer-shaped morphs than from enclosed to projected morphs or between enclosed and projected morphs (Fig. 1). Fig. 3 and Table 4 show the relative likelihood among three categories of floral traits on each of 17 focal nodes of the Bayesian consensus tree. Among these 17 nodes, the likelihood analysis significantly supported the presence of projected (nodes 1–8) or enclosed (node 9) morphs over saucer-shaped morph in the nodes 1–9. This implies that saucer-shaped flowers arose at least four times in *Heucherina*, given the phylogenetic relationships of saucer-shaped

Table 3  
Floral visitors of each *Heuchera* species and the body location on which pollen load attaches

Plant species	Floral visitor species	N	Location of pollen load (frequency)
<i>Heuchera cylindrica</i>	<i>Bombus</i> spp. (Hymenoptera, Apidae)	0	—
<i>H. micrantha</i>	Unidentified Diptera 1 (Syrphidae)	0	—
<i>Mitella acerina</i>	<i>Coelosia fuscicauda</i> Okada (Mycetophilidae)	31(28)	Head (89%), mouthpart (61%), coxa (11%)
	<i>Boletina</i> spp. (Mycetophilidae)	10(7)	Head (57%), mouthpart (71%), coxa (14%)
	<i>Gnoriste mikado</i> Okada (Mycetophilidae)	6(5)	Mouthpart (60%), coxa (80%)
	<i>Erioptera</i> sp. (Tipulidae)	1(1)	Head (100%)
<i>M. breweri</i>	<i>Boletina</i> spp. (Mycetophilidae)	SJ:12(12)	Head (25%), mouthpart (92%), coxa (8%)
	<i>Rhamphomyia</i> sp. (Empididae)	SJ:1(0)	—
<i>M. caulescens</i>	<i>Allodia</i> spp. (Mycetophilidae)	5(3)	Coxa (100%)
	<i>Boletina</i> sp. (Mycetophilidae)	1(1)	Coxa (100%)
	<i>Mycetophila</i> sp. (Mycetophilidae)	2(0)	—
	<i>Rhamphomyia</i> sp. (Empididae)	2(0)	—
<i>M. furusei</i> var. <i>furusei</i>	<i>Gnoriste mikado</i> (Mycetophilidae)	MS:1(1), FN:6(6)	Mouthpart (100%)
<i>M. furusei</i> var. <i>subramosa</i>	<i>Gnoriste mikado</i> (Mycetophilidae)	K:4(4), DA:5(4), AS:7(7), A:4(4), TR:1(1)	Mouthpart (100%), coxa (25%)
	<i>Rhamphomyia</i> sp. (Empididae)	AS:1(1)	Head (100%)
	Unidentified Diptera 2 (Empididae)	AS:1(1)	Mouthpart (100%)
<i>M. integripetala</i>	<i>Boletina</i> spp. (Mycetophilidae)	21(21)	Head (100%), mouthpart (86%), thorax (86%), coxa (81%)
	<i>Dolophilodes</i> sp. (Philopotamidae; Trichoptera)	1(1)	Abdomen (100%)
<i>M. japonica</i> Eastern strain	<i>Boletina</i> spp. (Mycetophilidae)	12(12)	Head (92%), mouthpart (92%), coxa (17%)
	<i>Coelosia fuscicauda</i> (Mycetophilidae)	7(7)	Head (71%), mouthpart (71%)
<i>M. japonica</i> Western strain	<i>Boletina</i> sp. (Mycetophilidae)	AM:3(3)	Mouthpart (100%)
	<i>Gnoriste mikado</i> (Mycetophilidae)	GI:3(2)	Mouthpart (100%)
<i>M. kiusiana</i>	<i>Boletina</i> sp. (Mycetophilidae)	T:1(1), I:1(1)	Mouthpart (100%)
	<i>Gnoriste mikado</i> (Mycetophilidae)	T:2(2), I:2(2)	Mouthpart (100%)
	<i>Mycetophila</i> sp. (Mycetophilidae)	I:1(1)	Thorax (100%)
	<i>Melanostoma</i> sp. (Syrphidae)	I:1(0)	—
	<i>Rhamphomyia</i> sp. (Empididae)	I:1(0)	—
	Unidentified Diptera 3	I:1(1)	Mouthpart (100%)
	Unidentified Diptera 4	I:1(1)	Mouthpart (100%)
	<i>Boletina</i> sp. (Mycetophilidae)	1(1)	Mouthpart (100%)
<i>M. koshiensis</i>	<i>Coelosia fuscicauda</i> (Mycetophilidae)	10(10)	Head (40%), mouthpart (90%), coxa (20%)
	<i>Gnoriste mikado</i> (Mycetophilidae)	1(1)	Mouthpart (100%)
	Unidentified Diptera 5 (Sciariidae)	4(4)	Head (100%), mouthpart (75%), thorax (25%), coxa (25%)
	<i>Melanostoma</i> sp. (Syrphidae)	1(0)	—
<i>M. nuda</i>	Unidentified Diptera 6 (Tipulidae)	2(1)	Head (100%), mouthpart (100%)
	Unidentified Diptera 7 (Tipulidae)	1(1)	head (100%), mouthpart (100%)
	<i>Allodia</i> sp. (Mycetophilidae)	RB:1(0), OK:2(2)	Head (100%), mouthpart (100%), coxa (100%)
	<i>Boletina</i> sp. (Mycetophilidae)	OK:2(2)	Head (100%), thorax (100%), coxa (100%)
<i>M. ovalis</i>	<i>Coelosia</i> sp. (Mycetophilidae)	OK:1(1)	Head (100%), thorax (100%), coxa (100%)
	<i>Rhamphomyia</i> sp. (Empididae)	OK:1(0)	—
<i>M. pauciflora</i>	<i>Mycetophila</i> sp. (Mycetophilidae)	0	—
<i>M. pauciflora</i>	<i>Allodia</i> spp. (Mycetophilidae)	HS: 6(3), IK1(1)	Coxa (75%), head (50%)
	<i>Boletina</i> spp. (Mycetophilidae)	Od:1(0), A:3(2), IK:1(1)	Coxa (67%), head (33%)
	<i>Coelosia fuscicauda</i> (Mycetophilidae)	HS:3(3), K:22(19), NI:1(1)	Coxa (95%), head (20%)
	<i>Baccha maculata</i> Walker (Syrphidae)	Od:1(0)	—
	<i>Melanostoma</i> sp. (Syrphidae)	Od:1(0)	—
	<i>Rhamphomyia</i> sp. (Empididae)	HS:1(0)	—
	<i>Myrmica</i> sp. (Formicidae)	HS:1(1)	Ventral surface (100%)
	Unidentified Hymenoptera 1 (Apidae)	Od:1(1)	Ventral surface (100%)
	Unidentified Hymenoptera 1 (Ichneumonidae)	Od:1(0)	—
	Unidentified Coleoptera	HS:2(2)	Ventral surface (100%)
	<i>M. pentandra</i>	<i>Boletina</i> sp. (Mycetophilidae)	SJ:8(8)
<i>M. stauropetala</i>	<i>Trichonta</i> sp. (Mycetophilidae)	SJ:1(0)	—
	<i>Greya mitellae</i> Davis & Pellmyr (Prodoxidae; Lepidoptera)	3(3)	Proboscis (100%)
	<i>Rhamphomyia</i> sp. (Empididae)	3(3)	Head (67%), mouthpart (67%)
	Unidentified Diptera 8 (Syrphidae)	1(0)	—

(continued on next page)



Table 3 (continued)

Plant species	Floral visitor species	N	Location of pollen load (frequency)
<i>M. stylosa</i> var. <i>stylosa</i>	<i>Gnoriste mikado</i> (Mycetophilidae)	6(6)	Coxa (67%), proboscis (67%)
<i>M. stylosa</i> var. <i>makinoi</i>	<i>Gnoriste mikado</i> (Mycetophilidae)	19(18)	Thorax (6%), proboscis (89%), mouthpart (67%), coxa (89%)
<i>M. yoshinagae</i>	<i>Baccha maculata</i> (Syrphidae)	5(1)	Head (100%)
	<i>Boletina</i> sp. (Mycetophilidae)	HK:4(4)	Mouthpart (100%)
<i>Tiarella polyphylla</i>	<i>Bacca</i> sp. (Syrphidae)	MR:1(1)	Ventral surface of head and thorax, and legs (100%)
	<i>Melanostoma</i> sp. (Syrphidae)	MR:6(6)	Ventral surface of head and thorax, and legs (100%)
	<i>Lasioglossum</i> sp. (Halictidae)	OK:1(1)	Ventral surface of head and thorax, and legs (100%)
<i>T. trifoliata</i> var. <i>unifoliata</i>	Unidentified Diptera 9 (Syrphidae)	—	—
<i>Tolmiea menziessi</i>	<i>Bombus</i> sp. (Hymenoptera, Apidae)	—	—
	<i>Gnoriste megarrhina</i> Osten Sacken (Mycetophilidae)	PC:4(4)	Head (25%), thorax (50%), coxa (100%)

The number of flower visitors captured with >10 pollen grains on their bodies were counted, and number of flower visitors with >40 pollen grains on particular body parts are shown in parentheses. The frequency of the body parts with >40 pollen grains were also calculated to clarify the characteristics of pollen attachment on insect body. In case multiple study sites are present for single plant taxon, the origin of the flower visitors are indicated with the abbreviations of study sites (see Table 2). Note that only observations of pollinator visits were made for *Heuchera cylindrica*, *H. micrantha*, *Mitella ovalis*, and *Tiarella trifoliata* va *unifoliata*.

species in the Bayesian consensus tree (Fig. 3). Moreover, 974 out of 1000 MCMC samples supported projected morph as the most-likely ancestral traits for the MRCA of all saucer-shaped species, whereas only 3 and 23 samples reconstructed enclosed and saucer-shaped morphs for the MRCA, respectively (Fig. 4). Therefore, multiple origins of saucer-shaped flowers were supported consistently after phylogenetic uncertainty was taken into account.

### 3.3. Pollination by fungus gnats is widespread both in Japan and Pacific Northwestern America

We surveyed natural pollinators of 28 *Heucherina* species and our data indicate that fungus gnats were principal pollinators of 18 plant species (13 in Japan and five in Pacific Northwestern USA and adjacent Canada [henceforth, PNW]), which always have greenish or brownish, dull-colored flowers (Fig. 5A–H and K–N). Pollen of these 17 species usually attach onto specific locations of the pollinator body (Fig. 5O–R and Table 3), implying consistent behavior of the pollinators on the flowers. Thirteen ingroup species (*Mitella* spp.) were visited by fungus gnats of several mycetophilid genera (Fig. 5A–H). On the other hand, five ingroup species, i.e., *Mitella furusei* var. *furusei* and var. *subramosa*, *M. stylosa* var. *stylosa* and var. *makinoi*, and *Tolmiea menziessi*, were visited and pollinated exclusively by a single species of fungus gnat, *Gnoriste mikado* Okada (four *Mitella* species in Japan; see online movie presented as Supplementary Material) or *G. megarrhina* Osten Sacken (*Tolmiea* in PNW), which have characteristic long proboscides (Fig. 5K–N). Fungus gnats never visited the remaining ten ingroup species (*Heuchera*, *Lithophragma*, *Tellima*, and some *Mitella* spp.) with showy, white-pink or cream-yellow flowers. Instead, bees, beeflies, hoverflies, and moths were their principal pollinators (Fig. 5I and J), as have previously been reported (Graenicher, 1907; Thompson and Pellmyr, 1992; Pellmyr et al., 1996; Weiblen

and Brehm, 1996; Segraves and Thompson, 1999; Nuismer and Cunningham, 2005).

## 4. Discussion

### 4.1. Phylogenetic relationships of *Heucherina* and its implication for taxonomy

Several earlier phylogenetic studies of *Heucherina* have been performed (e.g., Soltis and Kuzoff, 1995; Kuzoff et al., 1999; Okuyama et al., 2005), and our analyses expand on them by integrating all major lineages into one relatively large data matrix for the first time. The results suggest important novel phylogenetic relationships among *Heucherina* species. For example, while a previous study supported monophyly of genus *Heuchera* (Soltis and Kuzoff, 1995), the present analysis did not support it possibly because of the inclusion of the anomalous *Heuchera glabra* (Fig. 2). Meanwhile, the monophyly of *Heuchera* + *Bensoniella* was supported only in our Bayesian analysis (Fig. 2), a result suggested in a previous study (Soltis and Kuzoff, 1995). These observations suggest that the monotypic *Bensoniella* may be derived from within *Heuchera* and should be included therein, rather than separating *Heuchera* into a few genera. However, this placement needs a more thorough evaluation perhaps with more molecular and/or morphological data added, as the taxonomic distinctness of *Bensoniella* from *Heuchera* is supported by careful analyses of karyotypes as well as morphology (Soltis, 1980, 1984, 1988). Another unexpected result is the split of *Heucherina* into the major clades A and B with reasonable bootstrap or Bayesian support (Fig. 2). An important finding in the present phylogenetic analysis is the circumscription of genus *Mitella*, which has long been suggested to be polyphyletic yet there has not been consistent agreement for its classification (Soltis and Kuzoff,

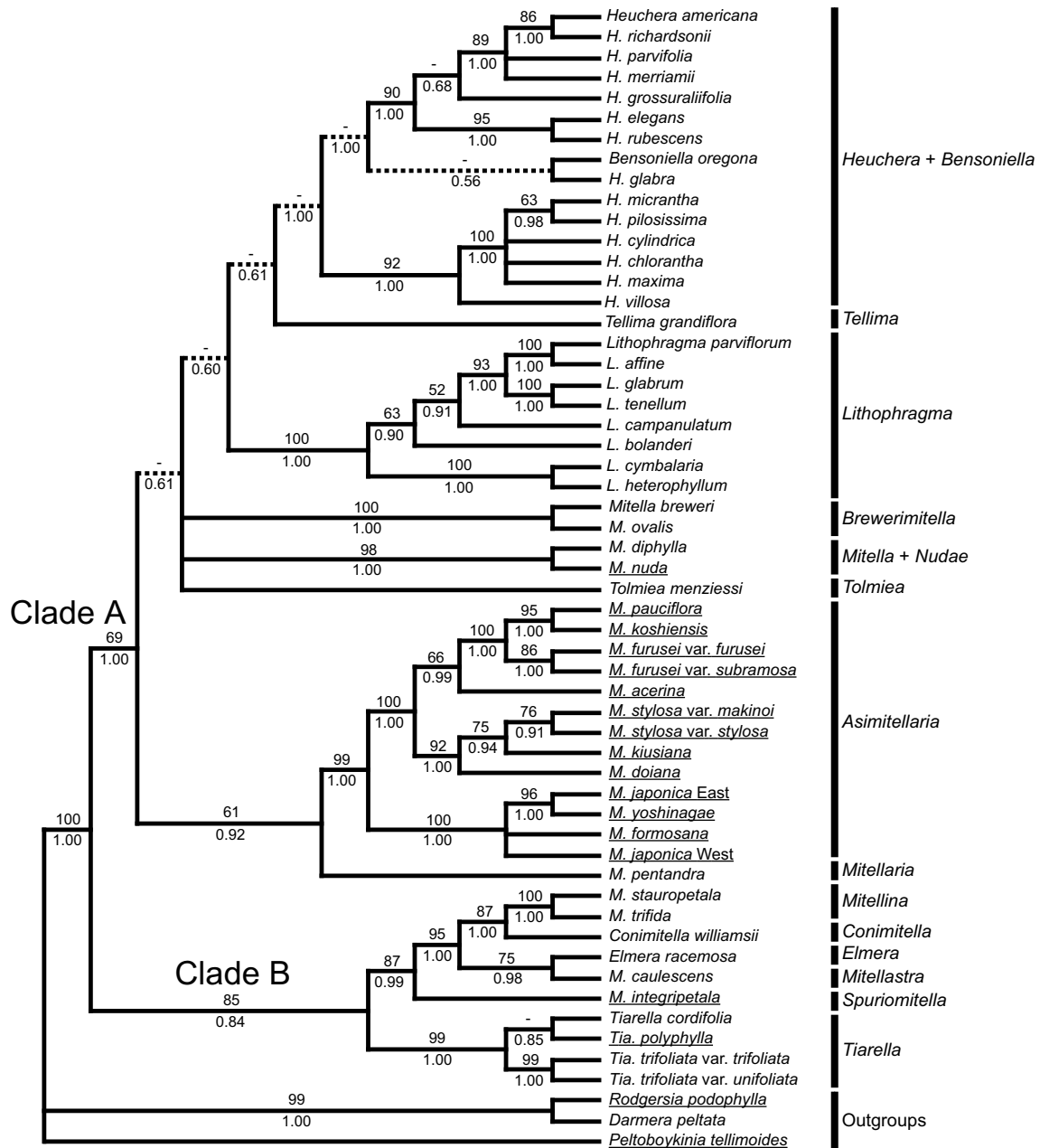


Fig. 2. The majority-rule consensus tree of 1000 trees derived from the Bayesian analysis of combined nuclear ribosomal ETS and ITS sequence data. Branches that collapse in the strict consensus tree of the parsimony analysis are shown with dashed lines. The numbers above branches indicate parsimony bootstrap support (>50% only), and those below branches indicate posterior probabilities. Underlined taxon labels indicate Asian species. Vertical bars to the right indicate clades that correspond to genera or conventional sections within genus *Mitella*.

1995; Soltis et al., 2001). In fact, the traditional genus *Mitella* was separated into six well-isolated clades usually with strong supports, i.e., *Brewerimitella*, *Mitella* + *Nudae*, *Asimitellaria* + *Mitellaria*, *Mitellina*, *Mitellastra*, and *Spuriomitella* (Fig. 2). It is noteworthy that the present phylogeny suggested close affinity of *M. pentandra* in PNW to Asian *Mitella* lineage *Asimitellaria* (Fig. 2), corresponding to their morphological synapomorphy of stamen arrangement that is opposite to the petals.

In summary, future taxonomic treatment would require the segregation of at least five genera from *Mitella*, and assessing the validity of including *Bensoniella* into *Heuchera*

and subdividing the whole *Heucherina* into two major groups.

#### 4.2. Multiple origins of saucer-shaped flowers as an adaptation to pollination by fungus gnats

Our extensive survey of pollination systems encompassing various species of *Heucherina* enabled us to examine the relationship between floral traits and pollination systems. Among the three categories of floral morphologies we defined (Fig. 1), “saucer-shaped” morphology was always associated with pollination by fungus gnats. Specif-

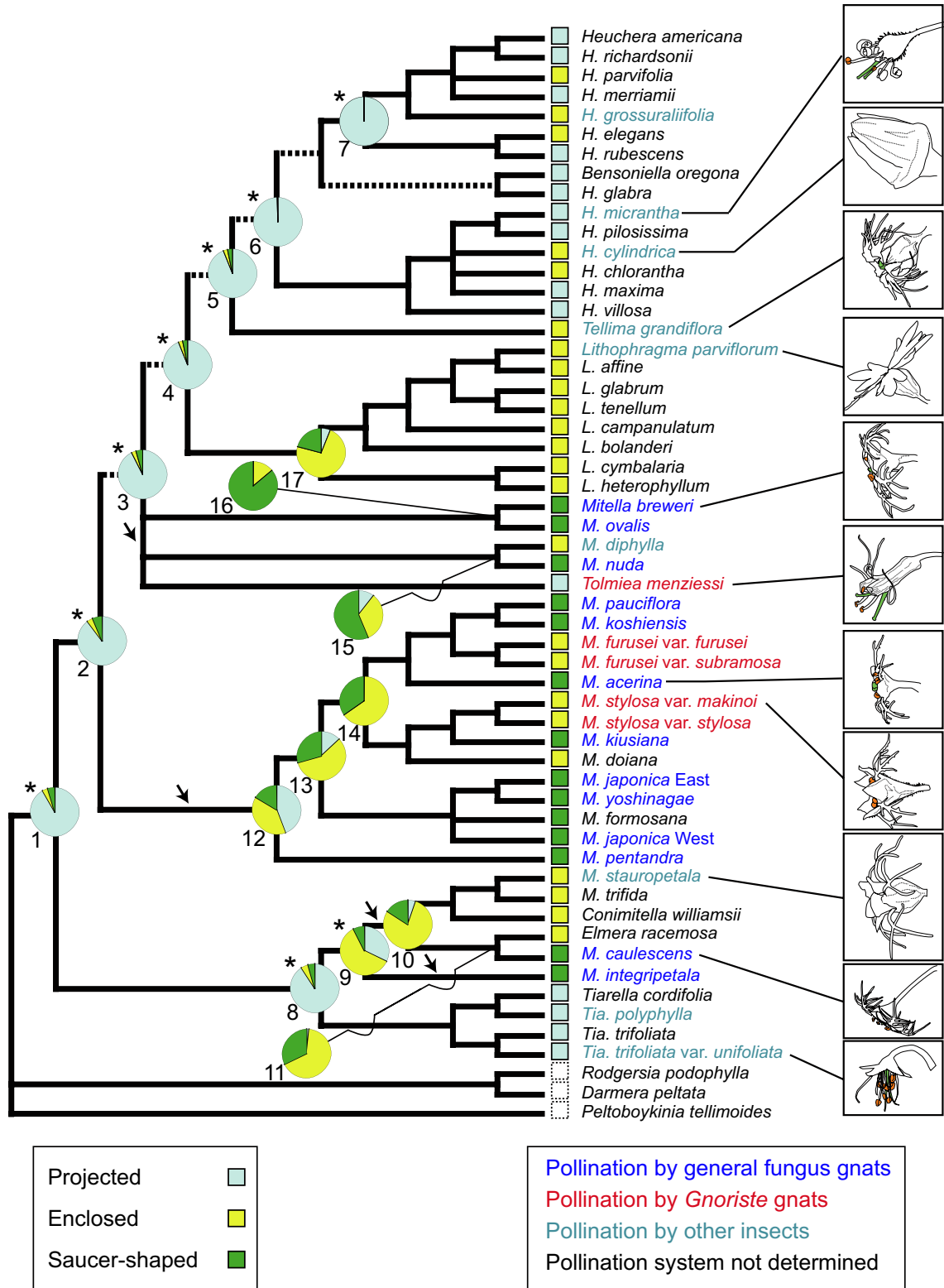


Fig. 3. Reconstruction of ancestral floral morphologies onto the Bayesian consensus tree of Heucherina. Pies illustrate relative likelihoods (local estimators) of the three possible states of floral morphologies at each node, i.e., projected (pale blue), enclosed (yellow), and saucer-shaped (green). Arrows indicate the lineages in which saucer-shaped flowers originated at least once independently. Names of species of which pollination systems were determined are colored in blue, red, or sky blue with each corresponding to pollination by general fungus gnats, *Gnoriste* gnats, and other insects, respectively. Lateral view of floral morphology of exemplar species are illustrated to the right of the tree with names of the represented species indicated. The anthers and pistils, when visible, are colored in orange and green, respectively in the illustrations. Branches that collapse in the strict consensus tree of the parsimony analysis are shown with dashed lines. Numbers shown below-left of pies correspond to the node numbers indicated in Table 4. Asterisks above pies indicate the nodes where projected (1–8) or enclosed (9) morphs were supported as significantly likely over saucer-shaped morph.

Table 4

Local log likelihood score of the three possible states of floral morphologies at each node indicated in Fig. 3

Node No.	Projected	Enclosed	Saucer-shaped
1	<b>-43.826182*</b>	-47.063617	-46.791756
2	<b>-43.826182*</b>	-46.941947	-46.475636
3	<b>-43.826182*</b>	-47.270938	-46.869827
4	<b>-43.826182*</b>	-47.357705	-47.170268
5	<b>-43.826182*</b>	-47.358312	-47.171892
6	<b>-43.826182*</b>	-49.689727	-49.710581
7	<b>-43.826182*</b>	-53.459945	-53.81134
8	<b>-43.826182*</b>	-46.799993	-46.859079
9	-44.953221	<b>-44.322341*</b>	-46.39302
10	-46.734351	<b>-44.046872</b>	-45.657071
11	-47.942099	<b>-44.222765</b>	-44.953092
12	<b>-44.517011</b>	-44.626475	-46.026448
13	-45.856035	<b>-44.374623</b>	-45.045872
14	-49.675001	<b>-44.255692</b>	-44.880595
15	-46.859079	-45.488936	<b>-44.357292</b>
16	-50.200805	-45.806244	<b>-43.969492</b>
17	-46.568539	<b>-44.106422</b>	-45.359818

The best score of the three in each node is shown in bold. Asterisks indicate significant support for projected or enclosed morphs over saucer-shaped morphs.

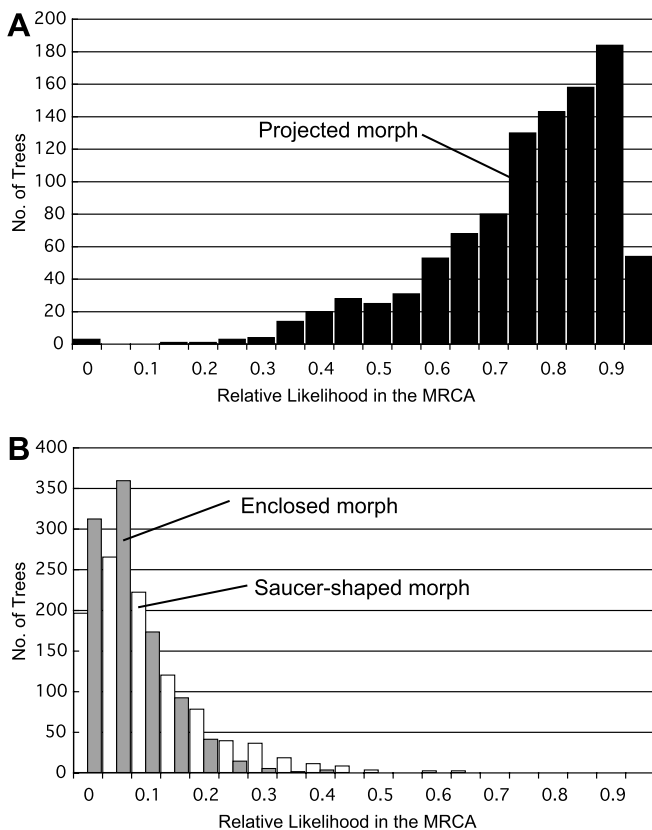


Fig. 4. Relative support for alternative floral traits in the MRCA of the extant saucer-shaped flowered species across the posterior distribution of trees from the Bayesian analysis. (A) Relative support for projected morph in the MRCA. (B) Relative support for enclosed (gray) and saucer-shaped (white) morphs in the MRCA. Note that there is no tree supporting enclosed or saucer-shaped morphs in the MRCA significantly, whereas 974 trees supported projected morph (of which 505 were significant) over saucer-shaped morphs.

ically, our study revealed that at least 13 of 14 described *Heucherina* species with saucer-shaped flower were almost exclusively pollinated by fungus gnats of the genera *Allo-dia*, *Boletina*, *Coelosia*, and *Mycetophila* (Table 3 and Fig. 5A–H), which do not have elongated mouthparts, during a grand total of 252 h of observation time. Meanwhile, the anomalous *Gnoriste* fungus gnats with elongated proboscides visited specific species having “projected” or “enclosed” flowers (Fig. 5K–N), which were not at all visited by other fungus gnats (Table 3). We confirmed the stability for these highly specialized pollination systems mediated by *Gnoriste* (here we use the terminology of specialized pollination system according to the concept of both ecological and evolutionary specialization, which were defined in Fenster et al., 2004) across years and study sites by careful observations of these five species (*Tolmiea* and *Mitella* spp.) during a total of 92 h over multiple study sites and years (Tables 2 and 3; additional 12 h observation of flowering *M. furusei* var. *subramosa* at Akame study site on May 1–2, 2006 recorded 102 visits of *G. mikado* only, Y.O., unpublished data). Notably, bees, hoverflies, beeflies, and moths, the important pollinators for the remaining *Heucherina* species (Fig. 5I and J), were seldom observed to visit the species pollinated by fungus gnats, especially those bearing saucer-shaped flowers (Table 3), even though our observations spanned long durations and encompassed various geographically isolated study sites (Table 2). Because the present phylogenetic analysis shows strong support for as many as four origins of the saucer-shaped morph in *Heucherina* (Fig. 3), the present data involve multiple evolutionary independent pairs of nonrandom association between saucer-shaped morphology and fungus-gnat pollination. Therefore, it is reasonable to conclude that the saucer-shaped floral morphology in *Heucherina* is an adaptation to pollination by fungus gnats with short mouthparts.

#### 4.3. Evolution of floral morphology in *Heucherina*

One of the advantages of maximum-likelihood approach with continuous Markov model (Pagel, 1994, 1999) for reconstructing ancestral states is that the method can test the suitable model for character evolution from the data set using stepwise likelihood-ratio tests. Although equally optimal models were possible for a character with three states (e.g., 41 models are possible for two-parameter model), our present data only resulted in a single model that fitted all of the 1000 MCMC samples of trees. As shown in Fig. 1, the resultant model suggested that evolution of floral morphology in *Heucherina* is strongly directional. For example, the model determined that the projected morph would shift easily toward enclosed morph but the reversal always difficult. Also, saucer-shaped morph would evolve easily only from enclosed morph, but character transition between projected and saucer-shaped morphs was always difficult. As a result, the evolutionary pathway toward



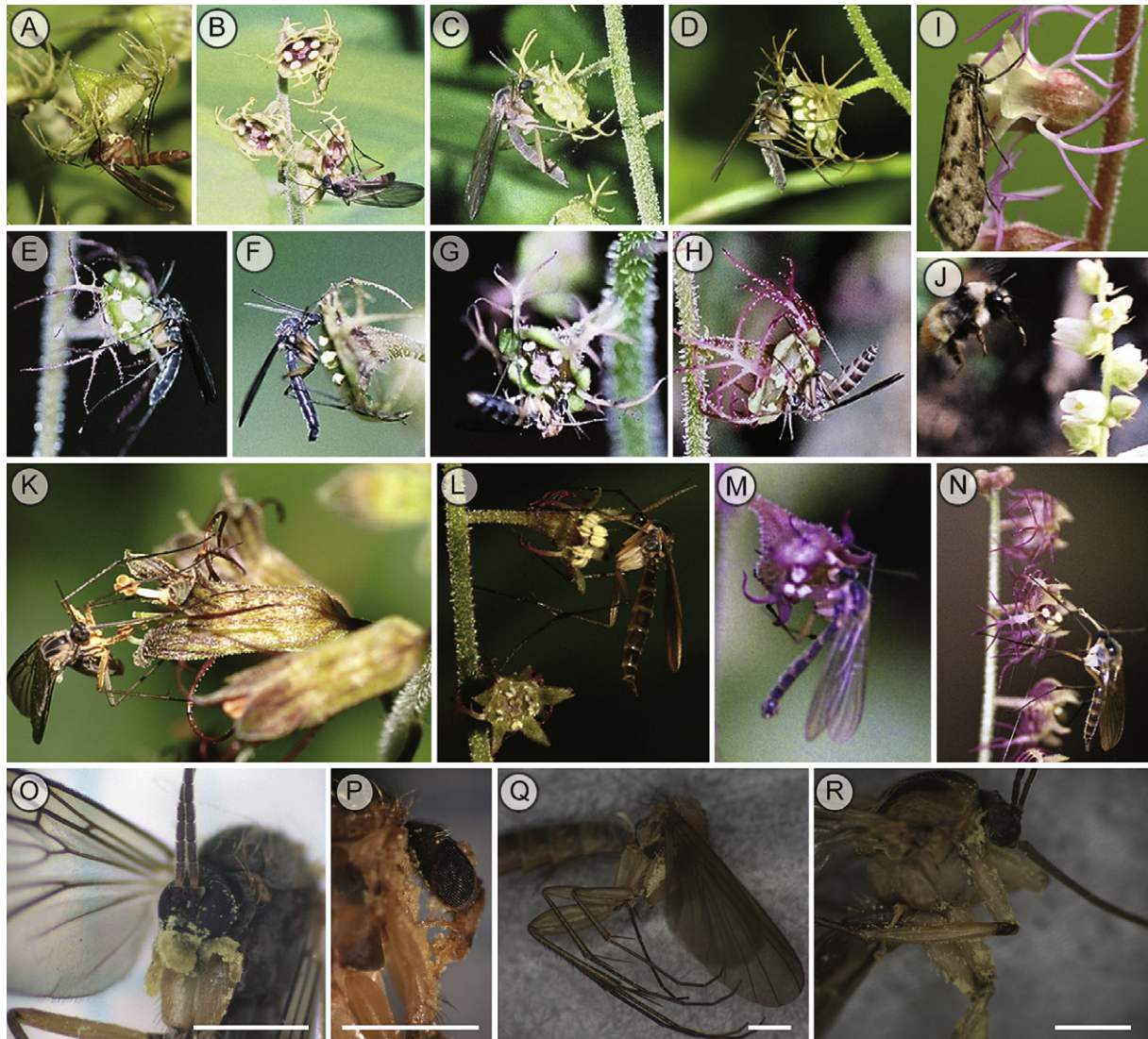


Fig. 5. Flowers of *Heucherina* plants and their pollinators. (A–H) Several unspecialized fungus gnat species pollinating the green flowers with exposed nectaries of (A) *Mitella caulescens*, (B) *M. pentandra*, and (C) *M. breweri* in PNW, and (D) *M. nuda*, (E) *M. japonica*, (F) *M. pauciflora*, (G) *M. koshiensis*, and (H) *M. yoshinagae* in Japan. (I) A prodoxid moth, *Greya mitellae* Davis and Pellmyr, visiting a white flower of *M. stauropetala*. (J) A bumblebee visiting a cream-yellow flower of *Heuchera cylindrica*. (K–N) A fungus gnat species, *Gnoriste megarrhina*, visiting a brown-purple tubular flower of (K) *Tolmiea menziesii* in PNW, and *Gnoriste mikado* visiting brown-purple campanulate flowers of (L) *M. furusei* var. *subramosa*, (M) *M. stylosa* var. *stylosa*, and (N) *M. furusei* var. *furusei*. (O–R) Pollinator fungus gnats collected on *Heucherina* flowers carrying numerous pollen grains on specific body regions; (O) *Boletina* sp. from *M. japonica* with pollen grains clustered on head and mouthparts, (P) *Boletina* sp. from *M. breweri* and (Q) *Allodia* sp. from *M. caulescens* having pollen grains on anterior coxae, and (R) *Gnoriste megarrhina* from *T. menziesii* having pollen grains on all the coxae. Bars = 1 mm.

saucer-shaped morph, which was shown to be an adaptation for fungus-gnat pollination, is suggested to be relatively simple. In turn, the projected morph is more likely to be ancestral, shifting toward the enclosed morph, and then evolving into the saucer-shaped morph (Fig. 1). There is no readily available explanation for such directionality in the floral evolution of *Heucherina*; it may be related to the genetic mechanisms and developmental constraints that regulate floral morphology, or may be affected by ecological and/or adaptive factors.

While useful, one should be cautious that all phylogenetic methods for ancestral state reconstruction are affected by several methodological problems. For example, the

method is unlikely to incorporate character states not observed in extant species. In addition, the application of a single model of character evolution onto the entire phylogenetic tree may be unrealistic in some cases (Pagel, 1999). Thus, the phylogenetic method is only useful for inferring evolutionary dynamics of the adaptive traits rather than to prove it, and the dynamics can definitely be established by direct identification and analyses of genes that actually regulates specific traits (see Igic et al., 2004, 2006). Because *Heucherina* species are known for their capability of artificial hybridization between relatively divergent species, even between different genera (Soltis and Kuzoff, 1995), future advances in molecular genetic



approaches may provide additional insights to pattern of floral evolution in Heucherina.

#### 4.4. Implication for multiple origins of the specialized pollination systems

Although our present analysis reconstructed the ancestral pollination system indirectly by coding floral morphologies, rather than pollination system itself, the observed pattern suggested that pollination by fungus gnats originated multiple times. This is based on the inference that the most recent common ancestor (MRCA) of Heucherina species with saucer-shaped flowers was not pollinated by fungus gnats, because the MRCA was supported to have projected floral morphology with high posterior probability ( $>0.97$ ; Fig. 4), and we found that fungus gnats do not visit most of the extant species with the latter floral morph except for *Tolmiea menziesii*. *Tolmiea* was pollinated only by the peculiar fungus-gnat species *Gnoriste megarrhina* with an extraordinarily long proboscis (Table 3 and Fig. 5K) that exactly fits the highly elongated calyx tube (Goldblatt et al., 2004). However, such specialization for the pollinator fungus gnats is unusual for the species with projected floral morph. The remaining species with projected floral morph have mostly white-pink or yellow, rather showy flowers with shallower calyces and are possibly pollinated by relatively unspecialized bees and hoverflies as was observed in four exemplar species (*H. micrantha*, *Tiarella cordifolia*, *T. polyphylla*, and *T. trifoliata*; Table 3 and Motten, 1986). There was a tendency for flowers pollinated by fungus gnats to have dull, greenish color, whereas those pollinated by bumblebees, hoverflies, or moths have much brighter, whitish or yellowish color.

It is also noteworthy that the enclosed floral morph was shown to have arisen in *Mitella* section *Asimitellaria* in Japan (Figs. 2 and 3). These species are pollinated by *Gnoriste mikado*, which has elongated mouthparts much shorter than those of *G. megarrhina* (Fig. 5L–N), and the plants always bear enclosed floral morph. Because these four plant species specialized to *Gnoriste* did not form a monophyletic group (Fig. 3), multiple transitions between enclosed and saucer-shaped morphs are necessary within the section *Asimitellaria*. Likewise, as *Tolmiea* and *Asimitellaria* were each specialized to highly differentiated *Gnoriste* species, it is very likely that such high specialization evolved independently in two distant regions (PNW and Japan).

Heucherina has been recognized as the lineage with the most diverse floral morphologies observed in Saxifragaceae (Soltis et al., 2001), and our study suggests that part of such floral diversity is associated with the repeated establishment of associations between the plants and pollinator fungus gnats. Because several Heucherina species are also known for their unusual pollination systems, such as highly specific brood-site pollination mutualisms (Thompson and Pellmyr, 1992; Pellmyr et al., 1996; reviewed in Sakai,

2002), Heucherina can be considered a promising model system for understanding the diversification of biotic pollination mechanisms.

#### 4.5. Fungus-gnat-flowers: an unrecognized pollination syndrome

In Heucherina, it is intriguing that both pollination by fungus gnats and the more specialized pollination by long-proboscis fungus gnats possibly have originated multiple times. Divergence of pollination systems are generally considered to occur either as the by-product of adaptation to different environments or, less frequently, as the adaptation for reproductive isolation per se (Grant, 1994). As in the case of other systems (Grant, 1993, 1994; Schemske and Bradshaw, 1999), the habitat changes most-likely account for the recurrent evolution of pollination by fungus gnats in Heucherina, because the plants pollinated by the fungus gnats and those pollinated by other insects do differ in their habitat preferences. The former are always associated with moist habitats along streams, whereas the latter are mostly found in much drier habitats such as forest edges, rocky soils, grasslands, and sagebrush deserts (Hitchcock and Cronquist, 1961; Okuyama et al., 2004). In addition, our preliminary observations suggest that larvae of the major pollinator genera *Boletina* and *Gnoriste* live among specific mosses and liverworts that grow in very moist riparian habitats together with the fungus-gnat-pollinated members of Heucherina (Hutson et al., 1980, and Y.O. and M.K., unpublished data). Accordingly, these fungus gnats are abundant in moist riparian woods (Søli et al., 1997) and thereby potentially reliable as pollinators only in such habitat. It is also noteworthy that the genus *Mitella*, which is clearly polyphyletic (Fig. 2), has long been recognized as a single genus. This may partly be because the independent adaptations for riparian moist habitats and resultant phenotypic convergence (e.g., greenish-colored, saucer-shaped flowers pollinated by fungus gnats and fruit capsules with “splash-cup” seed dispersal mechanisms, see Savile, 1975; Okuyama et al., 2004) across at least four different clades have masked the true phylogenetic relationships of the plants.

Because plants specialized to pollination by fungus gnats are quite uncommon, it was surprising that pollination by these insects is widespread and common among several clades within Heucherina in two distant biogeographic regions, viz. Japan and PNW. Fungus gnats as pollinators have usually been discussed under the context of “deception” syndromes (Vogel, 1978; Proctor et al., 1996; Vogel and Martens, 2000; Larson et al., 2001), in which the insects confuse the flowers as brood sites (sapromyophily, Proctor et al., 1996) or mating partners (Blanco and Barboza, 2005). In contrast, the plant-pollinator partnership between Heucherina plants and fungus gnats is based on a reward systems (Goldblatt et al., 2004; Okuyama et al., 2004). Moreover, Heucherina was also shown to have experienced parallel floral adaptations for the pollination sys-

tem, further indicating that these insects are very important as pollinators. Labandeira (2005) also recognized the importance of fungus gnats as pollinators with the description of “midge flowers” as small, white, purplish or green, hermaphroditic, mostly zygomorphic flowers with hidden rewards, which are pollinated by a vast spectra of basal dipteran families such as Culicidae, Mycetophilidae, Sciaridae, Cecidomyiidae, Ceratopogonidae, Simuliidae, and others (see also Larson et al., 2001; Yuan et al., 2007). Although there are some common characteristics in the saucer-shaped *Heucherina* flowers with Labandeira’s description (e.g., purplish or green color), the *Heucherina* species are unique in that they almost obligately depend for their pollination on specific genera of mycetophilid fungus gnats.

Here we propose that the pollination of reward-bearing flowers by fungus gnats constitutes an important mode of pollination in very moist habitats in temperate forests. The notion of a pollination syndrome is based on natural selection and convergence acting on floral traits exerted by a specific functional group of pollinators (Fenster et al., 2004). The only pollinators of some *Heucherina* plants, the fungus gnats, can be a distinct functional group, because they have relatively similar body shape and size, diurnal or crepuscular activities, foraging behavior, and habitats, which differ from those of most larger dipterans. Many fungus gnat species are only active at dusk and dawn (Søli et al., 1997), and this may be one reason why their role in pollination long has been overlooked. Two exceptions are specialized pollination of rewarding flowers by fungus gnats in two monocots, *Scoliopus* (Ackerman and Mesler, 1979) and *Listera* (Mesler et al., 1980). We have also found some other, possible cases of pollination by fungus gnats in distantly related plants, such as *Disanthus* (Hamamelidaceae) and *Ribes* (Grossulariaceae) in Japan (M.K. and Y.O., unpublished data). The flowers of *Bolandra oregana* (Saxifragaceae) and *Stenanthium occidentale* (Melanthiaceae) in PNW have close phenotypic similarities to *Tolmiea*, suggesting potential presence of a similar pollination system. The common features of these plants are their green or purple flowers sometimes with linear petals, saucer-shaped morphologies, and occurrence in moist habitats often along streams. Taken together, the “fungus-gnat-flowers” must have also evolved multiple times outside *Heucherina* in the temperate region. Hence, it can be treated as another example of pollination syndrome, although most of the phenotypic traits associated with the insects remain unclear. Because fungus gnats are prevalent in the temperate regions (Søli et al., 1997), extensive field studies may shed light on the general importance of these insects as selective agents of floral evolution.

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#### Appendix A. Supplementary data

Supplementary movie associated with this article can be found, in the online version, at [doi:10.1016/j.ympev.2007.09.020](https://doi.org/10.1016/j.ympev.2007.09.020).

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