

Adaptive Species Differentiation and Population Uniformity in *Viola* Species Sharing Similar Geographical Distribution but Differing Habitat Preferences

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Abstract

Selection of favorable alleles is sufficient to maintain species cohesion even with low levels of gene flow. Here, in the study of two sister species growing in contrasting restricted ecological areas, we assess whether their divergent traits have been subject to uniform selection among populations. Two different analyses were performed to evaluate the relative importance of selection and drift. First, we compared F_{ST} and P_{ST} indexes, analogous to Q_{ST} , in eleven populations each of *Viola eizanensis* and *V. chaerophylloides*. P_{ST} was computed for two reproductive traits, two leaf traits and two allocation traits. Second, we compared the observed P_{ST} with the expected distribution of P_{ST} under neutrality generated by phylogeny-based simulation assuming the Brownian motion model for trait evolution. These analyses indicated that spring leaf mass per area and seed number per capsule were divergent between species and uniform among populations. We suggest that these candidate traits are associated with ecological speciation. We also show that uniform, not divergent, selection among populations is probably common in both species. This result suggests that uniform selection maintaining similarity of traits among populations is a more dominant force than divergent selection in species growing in restricted ecological areas.

Keywords: Brownian motion model, ecological speciation, P_{ST} , Q_{ST} , sister species; species cohesion

1. Introduction

The maintenance of species cohesion has been of continued interest in evolutionary biology. In the classic view, evolutionary biologists regard species as evolutionary units that are defined as groups of interbreeding individuals isolated from other such groups (Mayr, 1963). Ehrlich and Raven (1969) challenged this view by reviewing evidence of gene flow in animal and plant populations that were generally too restricted to maintain species integration. Since this seminal paper, many efforts have been made to estimate gene flow in the wild and evaluate its role in species cohesion (e.g. Ellstrand & Elam, 1993; Levin, 2000; Slatkin, 1985). By reviewing both recent estimates of gene flow and analyzing the strength of selection on phenotypic traits, Rieseberg and Burke (2001) and Morjan and Rieseberg (2004) reached two key conclusions. First, there are numerous species that lack sufficient gene flow to prevent divergence, although gene flow is often considerably greater than suspected from earlier studies. Second, most selection coefficients for leading QTLs associated with interspecific differences are high enough for advantageous alleles to spread across the range of a species. Based on this evidence, they argued that advantageous QTLs are likely to contribute to species cohesion, even with a very low level of gene flow. However, their calculations of selection coefficients for QTLs were crude, probably resulting in an overestimation of the strength of selection. Further studies are required to demonstrate how selection on QTLs can be effective to maintain species integration under low levels of gene flow.

Comparative analysis among lineages of both phenotypic variation and molecular polymorphism of putatively neutral marker genes can provide an alternative effective approach to evaluate the role of selection in maintaining species cohesion. In this approach, the level of lineage divergence in neutral marker genes, quantified as F_{ST} (Wright, 1951), is compared with an analogous measure of phenotypic characters, Q_{ST} (Spitze, 1993). There are three possible outcomes from this comparison. First, if $F_{ST} < Q_{ST}$, then this implies that the degree of differentiation in quantitative traits exceeds the level achievable by genetic drift alone. In other words,

divergent natural selection favoring different phenotypes in different lineages is most likely to have been involved to achieve this differentiation. Second, if $F_{ST} \approx Q_{ST}$, this implies that the observed degree of differentiation in quantitative traits can be explained by genetic drift alone. The third possible outcome is $F_{ST} > Q_{ST}$. This implies that observed degree of lineage differentiation is actually smaller than what would be expected under genetic drift alone. In other words, uniform natural selection favoring the fittest phenotype probably acted on the traits.

Most comparative studies of F_{ST} and Q_{ST} have shown that the degree of differentiation in quantitative traits (Q_{ST}) typically exceeds that observed in neutral marker genes (F_{ST}) (e.g. Leinonen, O'Hara, Cano, & Merilä, 2008; McKay & Latta, 2002; Merilä & Crnokrak, 2001). These studies suggest a prominent role for natural selection in accounting for patterns of quantitative trait differentiation among contemporary populations. In several other studies, Q_{ST} and F_{ST} values did not differ significantly, suggesting that genetic drift alone was a sufficient explanation for the differentiation observed (e.g. Jorgensen, Richardson, & Andersson, 2006; Lascoux, Thorsen, & Gullberg, 1996; Widén, Andersson, Rao, & Widén, 2002). Finally, other studies in which F_{ST} exceeded Q_{ST} (e.g. Baruch, Nassar, & Bubis, 2004; Edmands & Harrison, 2003) suggested the footprints of uniform selection. Among this latter group particularly lie some plant species that have restricted ecological niches. Examples include *Scabiosa canescens* (Waldmann & Andersson, 1998), *Liatris scariosa* var. *novae-angliae* (Gravuer, von Wettberg, & Schmitt, 2005), *Cedrela odorata* (Navarro et al., 2005), *Thlaspi caerulescens* (Jimenez-Ambriz et al., 2007) and *Brassica insularis* and *Centaurea corymbosa* (Petit et al., 2001). Petit et al. (2001) suggested that restricted ecological niches could cause species to experience homogenous selection pressures, resulting in a lower Q_{ST} . However, all of those studies compared Q_{ST} and F_{ST} within a single plant species. Here, we studied a closely-related species pair, *Viola eizanensis* (Makino) Makino that prefers more shaded habitats, and *V. chaerophylloides* (Regel) W. Becker var. *sieboldiana* (Maxim.), which prefers more open habitats. We tested the prediction that species differences apparently adaptive to habitat differences were subject to divergent selection between species and uniform selection among populations within each species. To test this prediction, we compared F_{ST} and P_{ST} indexes, analogous to Q_{ST} . This approach would be error-prone because of the downward bias of P_{ST} , but P_{ST} based on measuring phenotypes grown in the common garden would be a suitable approach for finding candidate traits that are most likely under heterogeneous selection (Whitlock, 2008).

In addition to the analysis of F_{ST} and P_{ST} , as described above, we performed a phylogeny-based simulation to generate the distribution of P_{ST} expected under neutrality. As the comparison between F_{ST} and Q_{ST} is assumed to be non-biased by maternal effects, environmental effects, migration rates, mutation rates and selection on marker genes (Edelaar & Bjorklund, 2011; Edelaar, Burraco, & Gomez-Mestre, 2011; Hendry, 2002; Merilä & Crnokrak, 2001; Whitlock, 2008), phylogeny-based analysis permits more accurate assessment of whether the neutral hypothesis can be rejected for particular traits. To detect non-Brownian traits, we applied the Brownian motion model for trait evolution following Freckleton and Harvey (2006). There are three possible outcomes from the comparison of observed P_{ST} with its expected neutral distribution. First, if P_{ST} exceeds the 95% confidence interval of its neutral distribution, divergent natural selection would be suggested. Second, if P_{ST} is included within such a confidence interval, the genetic drift hypothesis is not rejected. Third, if P_{ST} is smaller than the confidence interval, uniform selection would be suggested.

Viola eizanensis and *V. chaerophylloides* var. *sieboldiana* provide an ideal opportunity for revealing the role of selection in species cohesion and differentiation. First, their sister relationship is supported by an unpublished ITS tree of *Viola* (Inoue, personal communication). Second, they are endemic to Japan, both having similar geographical distributions and altitudinal ranges (Hama, 2002; Igari, 1996). Third, their genetic structure and phylogenetic relationships were determined in our previous study (Toyama & Yahara, 2009). Fourthly, they are similar in most other ecological traits being small perennial herbs that produce chasmogamous (CH) flowers in spring and cleistogamous (CL) flowers in summer and autumn, and propagate only by elaiosome-bearing seeds dispersed by ants. They do, however, differ in habitat preference, *V. eizanensis* prefers more shaded places in temperate forests, and *V. chaerophylloides* var. *sieboldiana* prefers more open areas in temperate grasslands (Hama, 2002; Igari, 1996). Reflecting this difference in habitat preference, apparent adaptive phenotypic differences between two species have been described (Toyama and Yahara, in prep.). For example, the dry weight of CH flowers relative to the above-ground plant weight was larger in *V. chaerophylloides* than in *V. eizanensis*, a difference that appears to be adaptive as pollinator visitations are more frequent in *V. chaerophylloides*. The two species also differ in the display size and dry mass of CH flowers, and weight and area of spring leaves, differences that appear to be adaptive to shaded or open habitats. To our knowledge, this is the first report to compare results of both an analysis of F_{ST} and P_{ST} and a phylogeny-based analysis, and also to examine the relative importance of natural selection and genetic drift to apparently adaptive traits by comparing

sister species.

The present study aims to test our hypothesis that divergent traits between two sister species growing in contrasting restricted ecological areas have been subjected to uniform selection among populations in each species. We address the following questions: 1) What traits are divergent between species? 2) What traits are uniform among populations? 3) Is uniform selection among populations common in species having a restricted ecological niche? 4) Is there any difference between the two analyses?

2. Materials and methods

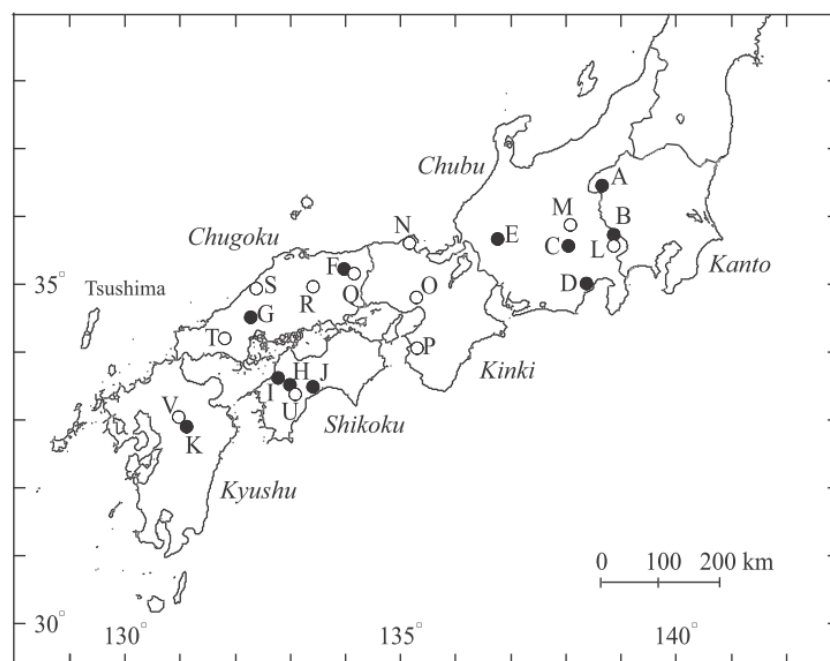
2.1 Study Species and Populations

The study species, populations and individuals were the same ones used in our previous study (Toyama & Yahara, 2009, Supplementary Table and Supplementary Figure). In July 2004, we collected about five individuals from each of the 22 populations that cover the distribution range of the two taxa in Japan. We collected individuals growing at least three meters apart to avoid any potential vegetatively reproduced individuals (although neither of these species are known to do this under ordinary environmental conditions). Only five individuals were sampled per population, which is an insufficient sampling, but individuals from each population clustered together (Toyama & Yahara, 2009). From our preliminary analyses, we judged that the number of populations examined was more important than the number of individuals per population. This decision was supported by a simulation study by Goudet and Büchi (2006) showing that a better estimate of Q_{ST} was obtained from many populations (> 20) with few families (5) rather than few populations with many families.

Supplementary Table. Sampled population codes and locations of two *Viola* species

Population code and location		Longitude(N)	Latitude (E)
<i>V. eizanensis</i>			
A	Mt. Ikaho, Ikaho-cho, Gunma-Prefecture	138.54	36.28
B	Mt. Ougi, Kaminohara-cho, Yamanashi-Prefecture	139.00	35.39
C	Osika Village, Nagano-Prefecture	138.06	35.34
D	Mt. Takakusa, Yaizu City, Sizuoka-Prefecture	138.19	34.54
E	Mt. Hunabuse, Motosu City, Gifu-Prefecture	136.41	35.38
F	Kagamino-cho, Okayama-Prefecture	133.57	35.11
G	Mt. Amida, Yuki-cho, Hiroshima-Prefecture	132.16	34.27
H	Oda-cho, Ehime-Prefecture	132.54	33.31
I	Mt. Saragamine, Kuma-cho, Ehime-Prefecture	132.54	33.43
J	Mt. Kuisi, Tosayama Village, Kochi-Prefecture	133.30	33.40
K	Takamori-cho, Kumamoto-Prefecture	131.08	32.52
<i>V. chaerophyllodes</i> var. <i>sieboldiana</i>			
L	Mt. Ougi, Kaminohara-cho, Yamanashi-Prefecture	139.01	35.39
M	Kayano plateau, Minowa-cho, Nagano-Prefecture	138.01	35.54
N	Ikari plateau, kyotango Cty, Kyoto-Prefecture	135.11	35.43
O	Mt. Hokusetsuohmine, Takaraduka City, Hyogo-Prefecture	135.19	34.51
P	Oumiisi plateau, Nokami-cho, Wakayama-Prefecture	135.20	34.06
Q	Nariwa-cho, Okayama-Prefecture	133.28	34.51
R	Anami Village, Okayama-Prefecture	134.06	35.13
S	Mt. Ootakayama, Oda City, Simane-Prefecture	132.25	35.03
T	Mt. Bahun, Shunan City, Yamaguchi-Prefecture	131.53	34.14
U	Mt. Kakusyougamori, Hayama Village	133.05	33.26
V	Aso-cho, Kumamoto-Prefecture	130.58	32.55

Modified from Toyama and Yahara (2009).



Supplementary Figure. Geographical locations of the 22 populations used in this study

Filled circles represent *V. eizanensis* (A-K), and open circles represent *V. chaerophylloides* var. *sieboldiana* (L-V). Letters refer to population symbols in Supplementary Table.

Modified from Toyama and Yahara (2009).

2.2 Greenhouse Experiment

This experiment was carried out in the Kyushu University nursery. After sampling, plants were planted in pots (12 cm diameter) and grown in the 50% shaded greenhouse. Pots were randomized every three days during the study. Quantitative traits were measured after the plants were grown in this common environment for at least nine months (from July 2004 to late March 2005) and measurements were performed from late March 2005 to June 2005.

We measured eight traits. CH and CL flower numbers were counted every three days and each flower was marked with colored tape (1 mm × 2 mm) to identify new flowers. For measurement of display size of CH flowers (DSCH, cm²), a picture of each flower was taken and processed together with a visual scale (1 cm²), which enabled measurement to the nearest 0.01 cm² using the Scion Image software ver. 4.0.2. The dry mass of CH flowers (DMCH, mg) was recorded using the first flowers produced, which were dried at 60°C and weighed with an electronic balance to the nearest 0.01 mg. Seed number per capsule of CL flowers (SN/CL) were counted for each flower. For weekly counting of leaf number, each leaf was marked with colored tape (1 mm × 2 mm) to enable the identification of new leaves. For the measurement of spring leaf area (SPLA, cm²), a measurement to the nearest 0.01 cm was obtained using the same technique as with the display size of CH flowers. To calculate spring leaf mass per area (SPLM/A, mg/cm²), a leaf with a known area was harvested from each individual plant, dried and weighed as previously described. We then calculated total investment in spring leaves (LEAF, mg) by multiplying SPLM/A with total SPLA for each individual. Total investment in CH flowers (W_{CH}) or CL flowers (W_{CL}) was calculated by multiplying the total flower number with the dried flower mass of each individual plant. The proportion of CH flower investment to the above-ground part of each plant (CH/AG) was calculated as

$$W_{CH}/(W_{CH}+W_{CL}+LEAF) \quad (1)$$

and the proportion of CL flower investment to the above-ground part of each plant (CL/AG) was calculated as

$$W_{CL}/(W_{CH}+W_{CL}+LEAF). \quad (2)$$

2.3 Quantitative Trait Analysis

Generally, there is a correlation among flower traits and a tradeoff among allocation traits. A principal

component analysis was performed to identify uncorrelated components in the chasmogamous (DSCH and DMCH) and allocation traits (LEAF, CH/AG and CL/AG) using R v2.3.1 (R Development Core Team, 2006). We analyzed the variation of the first principal component for chasmogamous traits and the first and second principal components for allocation traits.

A variance component analysis with species, populations nested within a species and individuals nested within a population as random effects was performed for each trait using the restricted maximum likelihood (REML) method. The estimates of σ_s^2 (genetic variance between species), σ_p^2 (genetic variance among populations within species) and σ_i^2 (genetic variance within populations) were used to quantify the level of species differentiation (P_{CT}) and population differentiation (P_{SC}) for each trait. P_{CT} and P_{SC} were calculated as follows, assuming the differences within and between populations or species were strictly genetic;

$$P_{CT} = \sigma_s^2 / (\sigma_s^2 + \sigma_p^2 + \sigma_i^2) \quad (3)$$

and

$$P_{SC} = \sigma_p^2 / (\sigma_p^2 + \sigma_i^2) \quad (4)$$

The 95% confidence interval for P_{CT} and P_{SC} was estimated by 10 000 bootstrap replications using R v2.3.1 (R Development Core Team, 2006).

To test the difference between P_{CT} (or P_{SC}) and neutral, we compared them with 95% confidence intervals for F_{CT} (or F_{SC}) estimated by AFLP (Toyama & Yahara, 2009). The use of five AFLP primer combinations on 121 individuals amplified a total of 533 fragments. Replications proved that the AFLP data was reliable (repeatability 99.2 % in *V. eizanensis*, 99.8 % in *V. chaerophylloides*). Analysis of molecular variance (Excoffier, Smouse, & Quattro, 1992) was used to investigate the partitioning of genetic variation with the HIERFSTAT package in R (Goudet, 2005). The confidence interval for each F -statistic was estimated by bootstrap over loci with 10 000 repeated samplings of the data. All analyses were performed in R v2.3.1 (R Development Core Team, 2006).

To generate the expected distribution of P_{CT} and P_{SC} under neutrality, we applied the phylogeny-based simulation technique of Freckleton and Harvey (2006) using R v2.3.1 (R Development Core Team, 2006). In this simulation, we first calculated P_{CT} and P_{SC} using the original data. Traits that had two or more data sets from one individual were analyzed using mean phenotypic values. Second, common ancestral trait values of X_k were reconstructed using a pair of adjacent tips (individual i and j) calculated as

$$X_k = (X_i/v_i + X_j/v_j) / (1/v_i + 1/v_j) \quad (5)$$

where v_i and v_j were the lengths of the branches leading to nodes i and j respectively. To account for statistical error (Felsenstein, 1985), the node below k was increased from v_k to

$$v_k + v_i v_j / (v_i + v_j). \quad (6)$$

Third, the standardized contrasts were calculated independently by their position on the phylogeny as follows;

$$|u_i| = |(X_j - X_k) / \sqrt{v_j + v_k}| \quad (7)$$

Fourth, the contrasts were randomized on the tree. Fifth, using contrasts and the above two formulae, a random value for the ancestral trait was computed at each node. The sign (positive or negative) of the second equation was assigned at random. This procedure was repeated until randomized values had been assigned to all nodes. Fifth, we calculated the simulated P_{CT} and P_{SC} . Finally, steps two to five above were repeated 10 000 times to estimate the empirical confidence interval of simulated P_{CT} and P_{SC} .

In the above simulation, we used a maximum parsimony (MP) tree instead of the neighbor joining (NJ) tree of Toyama and Yahara (2009) with the number of substitutions per branch as branch length. The MP tree showed an almost identical topology to the NJ tree.

This simulation is associated with calculation errors when there are polytomies on the tree. We made some assumptions about the unknown branching patterns of multiple nodes to permit analysis. Because *V. eizanensis* and *V. chaerophylloides* do not reproduce vegetatively under ordinary environmental conditions, it is unlikely that polytomies would be due to clonal individuals. Therefore, we set the virtual node to having only one branch length. We randomly selected a branching pattern from all possible topologies in each repeat simulation.

3. Results

Principal component analysis indicated that 77.1% of variation in CH flower traits could be explained by the first principal component (PC1). PC1 and PC2 could also explain 51.7% and 33.3%, respectively, of the variation in allocation traits (Table 1, Figure 1). In the allocation traits, PC1 separates plants with larger vegetative organs from plants with larger reproductive outputs, and PC2 discriminates plants with a larger allocation of CL flowers

from plants with a larger allocation of CH flowers. Phenotypic variation in each quantitative trait is shown in Figure 2.

Table 1. Contribution of CH flower and allocation traits to the principal components of phenotypic variation

Trait	Coefficient		
	PC1	PC2	PC3
CH flower			
CHDS	-0.707	0.707	-
DMCH	-0.707	-0.707	-
Variance explained	0.771	0.229	-
Allocation			
Allocation	-0.707	3.359×10^{-5}	-0.707
LEAF	-0.707	3.359×10^{-5}	-0.707
CH/AG	0.529	-0.663	-0.529
CL/AG	0.469	0.749	-0.469
Variance explained	0.517	0.333	0.150

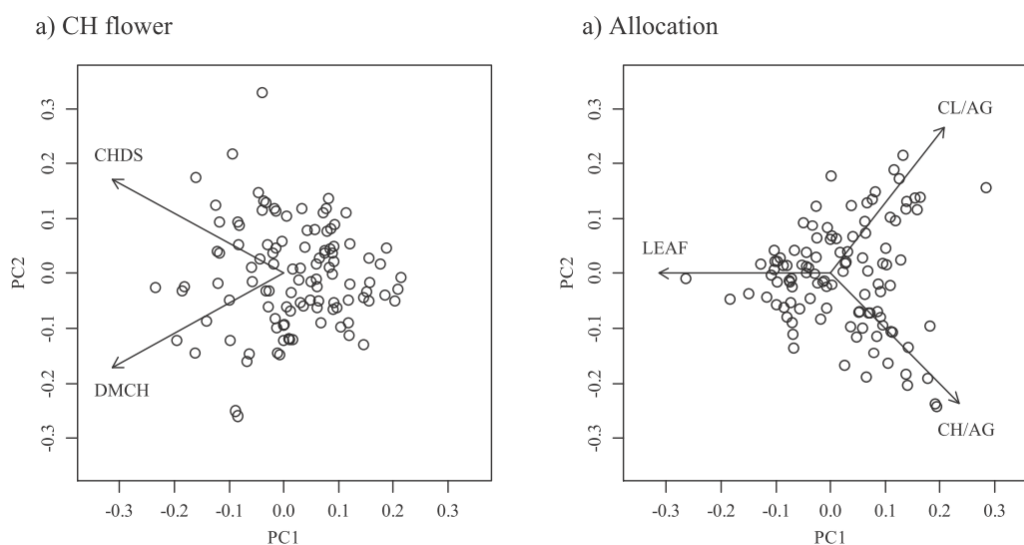


Figure 1. Principal component analysis of CH flower and allocation traits

A plot of the first and second principal components of the (a) CH flower traits and (b) allocation traits. Each circle represents an individual. Arrows indicate the contribution of the trait to the principal components.

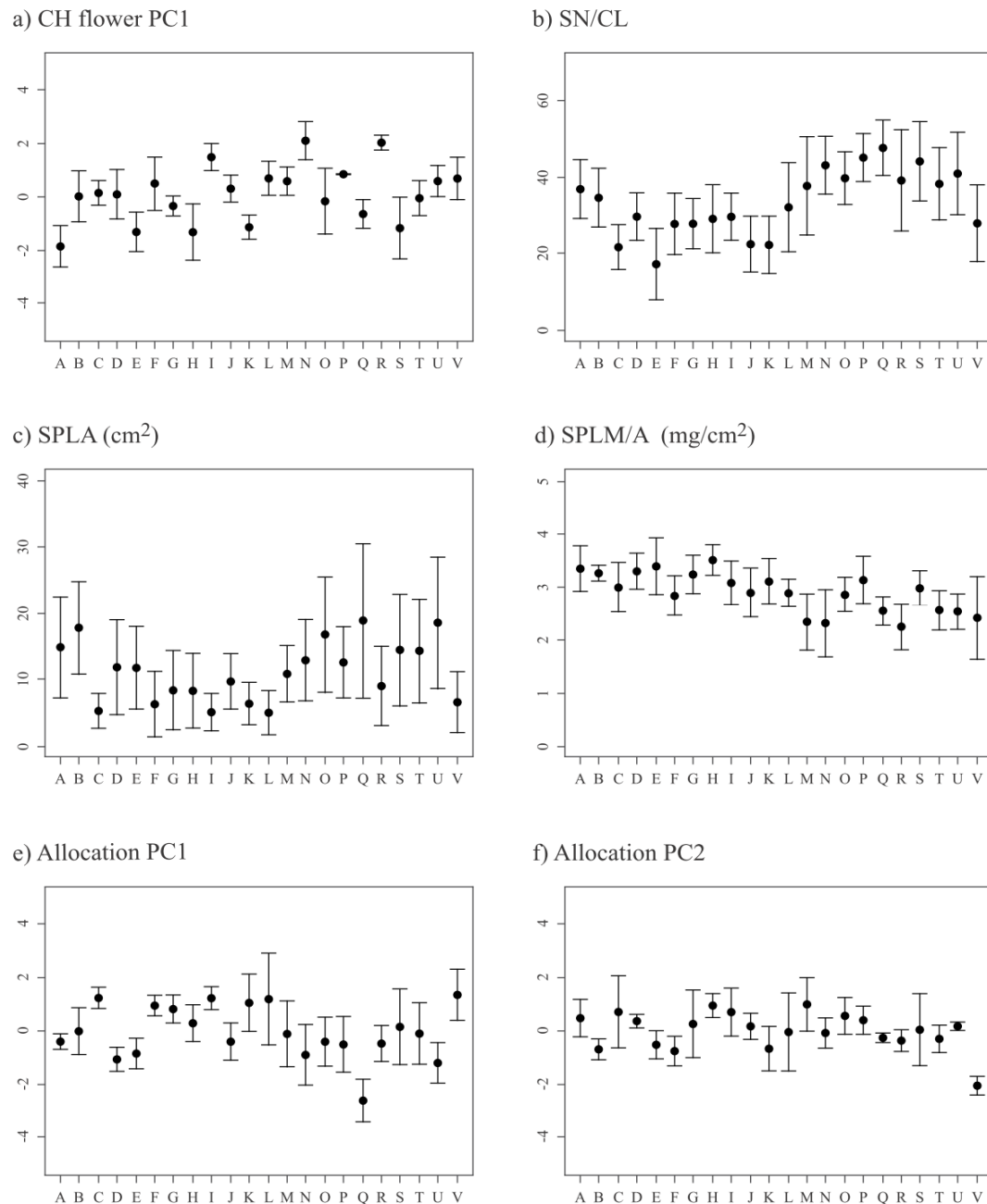


Figure 2. Phenotypic variation of six traits in *V. eizanensis* (populations A-K) and *V. chaerophylloides* (populations L-V)

The mean $\pm$ S.D. of (a) PC1 for CH flower traits, (b) seed number per capsule, (c) spring leaf area, (d) spring leaf mass per area, (e) PC1 and (f) PC2 for allocation traits is shown. Letters indicate population symbols referred to in Toyama and Yahara (2009) and Figure S1.

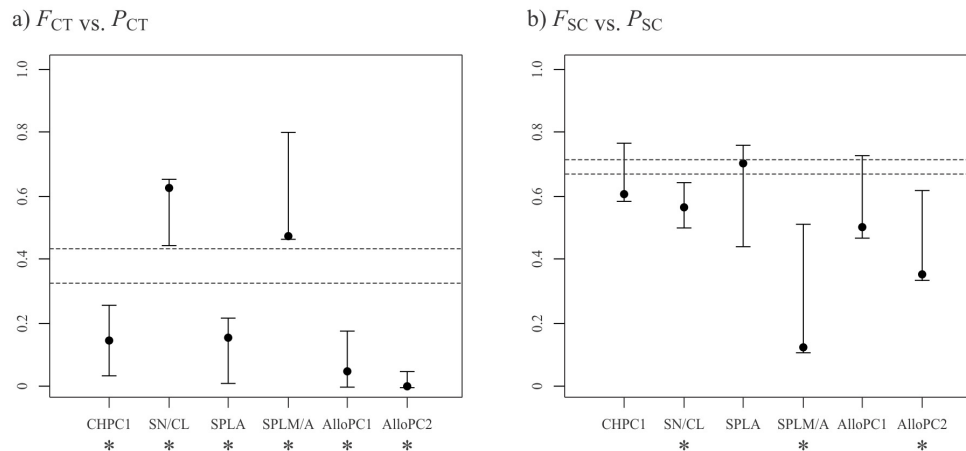


Figure 3. Confidence intervals for (a) F_{CT} and P_{CT} , (b) F_{SC} and P_{SC}

Each dot represents the observed P_{CT} or P_{SC} value and each bar represents the 95% confidence interval. The upper and lower horizontal dotted lines indicate the 95% confidence intervals of F_{CT} and F_{SC} . Asterisks indicate that the 95% CIs do not overlap.

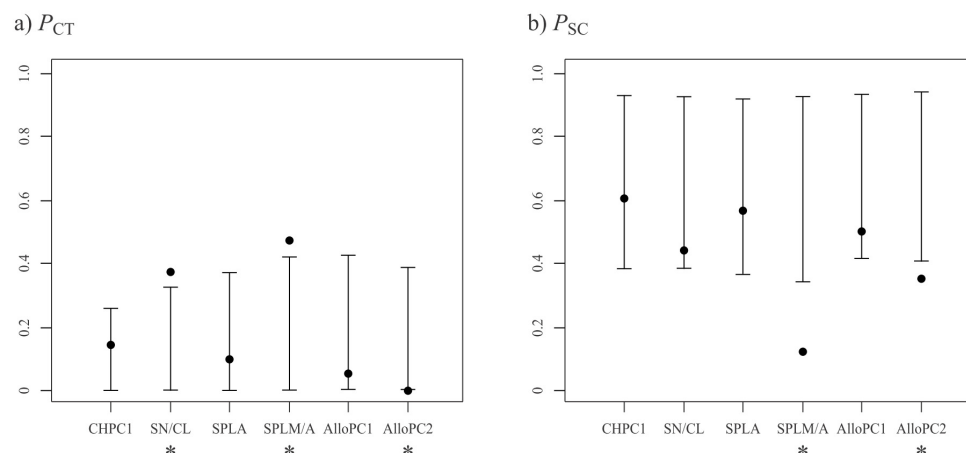


Figure 4. Comparison between (a) observed P_{CT} and the distribution of P_{CT} expected under neutrality and (b) observed P_{SC} and expected P_{SC}

Each dot represents an observed value and each bar represents the 95% of confidence interval of its neutral distribution. Asterisks indicate that the observed value does not overlap with the 95% CI for neutrality.

Estimates of P_{CT} ranged from 3.28×10^{-9} (PC2 for allocation traits) to 0.631 (SN/CL) (Figure 3a). By comparing the 95% CI of P_{CT} with that of F_{CT} , it was shown that P_{CT} was significantly higher in two traits: SN/CL and SPLM/A, and significantly lower in four traits: PC1 for CH flower traits, SPLA, and PC1 and PC2 for allocation traits. Estimates of P_{SC} ranged from 0.121 (SPLM/A) to 0.704 (SPLA) (Figure 3b). By comparing the 95% CI of P_{SC} with that of F_{SC} , it was shown that P_{SC} was significantly lower in three traits; SN/CL, SPLM/A and PC2 for allocation traits.

The 95% confidence intervals of P_{CT} expected under neutrality are shown in Figure 4a. A significantly higher P_{CT} value than the neutral expectation was observed in SN/CL and SPLM/A, and a significantly lower P_{CT} value was observed in PC2 for allocation traits. The 95% confidence intervals of P_{SC} expected under neutrality are shown in Figure 4b. A significantly lower P_{SC} value than the neutral expectation was observed in SPLM/A and PC2 for allocation traits.

4. Discussion

4.1 Adaptive Divergence between Species and Phenotypic Uniformity among Populations

Ecological speciation is defined as the evolution of reproductive isolation between populations by divergent natural selection arising from differences between ecological environments (Coyne & Orr, 2004; Schluter, 2001). In this study, we were able to identify candidate traits associated with ecological speciation. *Viola eizanensis* and *V. chaerophylloides* are separated by their differing light preference, and comparative analysis between F_{ST} and P_{ST} values and phylogeny-based simulation analysis supported the observation that two of six traits (SN/CL and SPLM/A) were subject to divergent selection between species and uniform selection among populations (Figures 3, 4).

Spring leaf dry mass per area values (SPLM/A) were larger for *V. eizanensis* than *V. chaerophylloides* (Figure 1d). This interspecific differentiation may be related to a difference in leaf longevity, as leaves having larger mass per area are expected to have greater longevity and are seen as adaptive for shaded habitats. This positive correlation between leaf mass per area and leaf longevity has been reported in many studies (e.g. Reich, Walters, & Ellsworth, 1992, 1997; Reich et al., 2003; I. J. Wright et al., 2005). Generally, in high light conditions, photoinhibition decreases plant productivity so that natural selection favors the production of new leaves. Conversely, in dark conditions the photosynthesis rate is low but stable so that natural selection favors the maintenance of leaves. This correlation between light conditions and leaf longevity has been previously reported (e.g. Sterck, 1999). However, further studies are needed to test whether the two species differ in leaf longevity as we predict.

For the number of CL seeds per capsule (SN/CL), *V. chaerophylloides* tends to have more seeds than *V. eizanensis* (Figure 2b). This interspecific differentiation may be related to the trade-off between seed number and size. In general, species producing many small seeds are considered to be superior colonizers, whereas species producing fewer large seeds are considered to be superior competitors (Coomes & Grubb, 2003). *V. chaerophylloides* grows in open and more disturbed grassland habitats and has more CL seeds over the growing season, a characteristic that may be advantageous for seed dispersal into newly available open habitats. On the other hand, *V. eizanensis*, grows in closed and more stable woodland habitats and produces fewer CL seeds, which are potentially better able to survive in shaded environments with correspondingly fewer resources, and withstand unfavorable conditions for seed germination and seedling growth. Such association between light conditions and seed number/size has been observed in some plant species (Foster & Janson, 1985; Grubb & Metcalfe, 1996; Hewitt, 1998).

Phenotypic plasticity in the above two traits could affect their ecological speciation. Recently, an individual-based simulation study showed that phenotypic plasticity could either enhance or degrade the process of ecological speciation depending on the timing (Thibert-Plante & Hendry, 2011). If the evolution of plasticity occurs before dispersal to different environments, it can increase the progress of ecological speciation. If plasticity occurs after dispersal, progress can be slowed because migrants are better suited to their new environments. Considering our findings, the SPLM/A and SN/CL traits, subject to divergent selection between species and uniform selection among populations, might represent evolved plasticity before the speciation process. There is no evidence to support this hypothesis, but it might be possible to confirm by examining the plastic response of the two species and/or common ancestral species in different light conditions. In summary, the two species we studied differ in two functional traits, spring leaf dry mass per area and the number of CL seeds per capsule, and these differences may have evolved as a result of their individual light requirements. Further studies are needed to reveal how these differences play a role in their respective speciation processes.

In contrast to the above findings, the other four traits were subject to uniform selection or genetic drift (Figure 3, 4). Particularly, PC2 for allocation traits was subject to uniform selection within and between species in both analyses (Figures 3, 4). This result suggests that a common plasticity mechanism has evolved for the allocation ratio of CH and CL flowers in the ancestor of both species. Theoretically, the reproductive system of CH and CL flowers evolved as a result of adaptation to variation in biotic and abiotic environments (Schoen & Lloyd, 1984). Many previous studies have shown that the proportions of CH and CL flower production are variable in response to light availability (Cheplick, 2006; Lecorff, 1993; Masuda & Yahara, 1994; Masuda, Yahara, & Maki, 2004; Mattila & Salonen, 1995; Schemske, 1978; Wilken, 1982), soil nutrient availability (Lecorff, 1993), soil moisture (Brown, 1952) and plant density (Wilken, 1982). However, further studies are required to elucidate whether the ratio of CH to CL flower investment is regulated by the same mechanism in many other cleistogamous species. For the other three traits (CHPC1, SPLA, AlloPC1), we couldn't detect a strong selection within or between species, although the comparative analysis between F_{ST} and P_{ST} was significant (Figures 3 and

4). These results suggest that genetic drift might have played the primary role in driving phenotypic differentiation.

4.2 Low Values of P_{SC} in the Restricted Ecological Niche of Species

The comparative analysis between F_{SC} and P_{SC} values and the phylogeny-based simulation showed that in both species P_{SC} was often lower than F_{SC} and its neutral distribution (Figures 3b, 4b). In no cases was the observed P_{SC} higher than the observed F_{SC} or expected P_{SC} under neutrality. These results imply that observed phenotypic differentiation among populations of the two species can be explained by random genetic drift or uniform selection rather than by divergent selection. This finding contrasts with previous reports where Q_{ST} was larger than F_{ST} (Leinonen et al., 2008; McKay & Latta, 2002; Merilä & Crnokrak, 2001).

This contrast may be due to the restricted ecological niches of the two species; adaptation to woodland and grassland habitats may be associated with homogenous selection pressures for phenotypic variation. Our lower P_{ST} result is consistent with findings in other ecologically restricted plant species (Grubb & Metcalfe, 1996) such as *Scabiosa canescens* (Waldmann & Andersson, 1998), *Liatris scariosa* var. *novae-angliae* (Gravuer et al., 2005), *Cedrela odorata* (Navarro et al., 2005), *Thlaspi caerulescens* (Jimenez-Ambriz et al., 2007) and *Brassica insularis* and *Centaurea corymbosa* (Petit et al., 2001). Thus, we suggest that the niche requirement of restricted species could act as uniform selection among populations.

4.3 Comparison between P_{ST} and Phylogeny-based Analyses

We applied two different methods to elucidate the relative importance of selection and drift. In all cases, phylogeny-based analysis was more conservative than P_{ST} analysis (Figures 3, 4). In Figures 3a and 4a, uniform selection was suggested for four traits by P_{CT} analysis, but only one trait in the phylogeny-based analysis. Similarly, in Figures 3b and 4b, uniform selection was suggested for three traits by P_{SC} analysis, but only two traits in the phylogeny-based analysis. There are three possibilities to explain this discrepancy. First, in comparative analysis between F_{ST} and P_{ST} , underestimated P_{ST} values could more easily result in apparent support for uniform selection than in phylogeny-based analysis. This study was not performed with progeny of experimental crosses, but with plants sampled from natural populations. In this case, non-additive genetic effects (e.g. dominance, maternal) could cause overestimation of genetic variance within populations and underestimation of Q_{ST} (Merilä & Crnokrak, 2001; Whitlock, 2008). However, dominance effects might be small because of the predominance of inbreeding in these two species (Goudet & Buchi, 2006). Second, linkage disequilibrium due to being highly selfing may also cause overestimation of F_{ST} . Our study assumed that the AFLP molecular markers are selectively neutral and unlinked to loci under selection. However, in partially selfing organisms, variation at neutral loci can be greatly influenced by selection acting on linked and even unlinked loci via genetic hitchhiking (Hedrick, 1980). Charlesworth et al. (1997) also demonstrated that, for subdivided populations, heterogeneous selection enhances F_{ST} values even at neutral loci distant to the selected locus. The AFLP mutation rate could also bias F_{ST} estimation. To our knowledge, there is no general mutational model for AFLP markers, but Edelaar et al. (2011) demonstrated that estimates of the differences between Q_{ST} and F_{ST} was biased depending on the mutation rate of the genetic markers used. We should await further studies on this topic before drawing conclusions. Thus, we must be careful in interpreting the results of comparative analysis between F_{ST} and P_{ST} . Third, a conservative policy in phylogeny-based analysis could also cause this discrepancy. As the distribution of P_{ST} expected under neutrality was performed under maximum parsimony criteria, it might be more difficult to detect uniform selection in the comparison with observed P_{ST} . Thus, there are some limitations in this study, but in areas where the results in two analyses are consistent sufficient data is presented for some initial conclusions.

5. Conclusion

Traits divergent between species and unified among populations are considered to be of particular importance in understanding the speciation process. In the present study, we detected adaptation in two traits in response to light preferences. In *V. eizanensis*, having larger leaf mass per area is expected to lead to greater leaf longevity and is a trait considered adaptive to shaded habitats. Likewise, the production of fewer seeds is expected to result in a higher seedling survival rate under restricted light conditions. Conversely, in *V. chaerophylloides*, smaller leaf mass per area is expected to ensure effective photosynthesis under higher light conditions, and having more CL seeds may be advantageous for seed dispersal into newly available open habitats. We have also demonstrated that the ratio of CH to CL flower investment is uniform both within and between species. To our knowledge, this is the first report to reveal the selection of allocation ratio between CH and CL flowers. Additionally, we have shown that low P_{SC} values are consistent with previous studies performed on species having a restricted ecological niche (Gravuer et al., 2005; Jimenez-Ambriz et al., 2007; Navarro et al., 2005; Petit et al., 2001;

Waldmann & Andersson, 1998). Therefore, we suggest that uniform selection is a more dominant force in these species maintaining uniformity of traits than has previously been suggested in recent reviews. Our finding supports the view that low gene flow species are held together primarily by the spread of advantageous alleles (Rieseberg and Burke 2001; Morjan and Rieseberg 2004). However, our calculations of P_{ST} based on measuring phenotypes grown in a common garden was biased downwards (Whitlock, 2008); therefore our analysis should be considered a first screen. To further test this view, mapping QTLs for the two traits divergent between species and unified among populations is a potential approach (e.g. Raeymaekers, Van Houdt, Larmuseau, Geldof, & Volckaert, 2007). *V. eizansensis*, and *V. chaerophylloides*, are inter-fertile and our on-going work with F2 hybrids of the two species will hopefully lead to a better understanding of the role of natural selection upon cohesion and differentiation of these species.

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References

- Baruch, Z., Nassar, J. M., & Bubis, J. (2004). Quantitative trait, genetic, environmental, and geographical distances among populations of the C(4) grass *Trachypogon plumosus* in Neotropical savannas. *Diversity and Distributions*, 10(4), 283-292. <http://dx.doi.org/10.1111/j.1366-9516.2004.00102.x>
- Brown, W. V. (1952). The Relation of Soil Moisture to Cleistogamy in *Stipa-Leucotricha*. *Botanical Gazette*, 113(4), 438-444. <http://dx.doi.org/10.1086/335732>
- Charlesworth, B., Nordborg, M., & Charlesworth, D. (1997). The effects of local selection, balanced polymorphism and background selection on equilibrium patterns of genetic diversity in subdivided populations. *Genet Res*, 70(2), 155-174. <http://dx.doi.org/10.1017/S0016672397002954>
- Cheplick, G. P. (2006). A modular approach to biomass allocation in an invasive annual (*Microstegium vimineum*; Poaceae). *Am J Bot*, 93(4), 539-545. <http://dx.doi.org/10.3732/ajb.93.4.539>
- Coomes, D. A., & Grubb, P. J. (2003). Colonization, tolerance, competition and seed-size variation within functional groups. *Trends in Ecology & Evolution*, 18(6), 283-291. [http://dx.doi.org/10.1016/S0169-5347\(03\)00072-7](http://dx.doi.org/10.1016/S0169-5347(03)00072-7)
- Coyne, J. A., & Orr, H. A. (2004). *SPECIATION*. Sunderland (MA): Sinauer Associates.
- Edelaar, P., & Bjorklund, M. (2011). If F(ST) does not measure neutral genetic differentiation, then comparing it with Q(ST) is misleading. Or is it? *Molecular Ecology*, 20(9), 1805-1812. <http://dx.doi.org/10.1111/j.1365-294X.2011.05051.x>
- Edelaar, P., Burraco, P., & Gomez-Mestre, I. (2011). Comparisons between Q(ST) and F(ST)-how wrong have we been? *Molecular Ecology*, 20(23), 4830-4839. <http://dx.doi.org/10.1111/j.1365-294X.2011.05333.x>
- Edmands, S., & Harrison, J. S. (2003). Molecular and quantitative trait variation within and among populations of the intertidal copepod *Tigriopus californicus*. *Evolution*, 57(10), 2277-2285. <http://dx.doi.org/10.1554/03-019>
- Ehrlich, P. R., & Raven, P. H. (1969). Differentiation of populations. *Science*, 165(3899), 1228-1232. <http://dx.doi.org/10.1126/science.165.3899.1228>
- Ellstrand, N. C., & Elam, D. R. (1993). Population Genetic Consequences of Small Population-Size - Implications for Plant Conservation. *Annual Review of Ecology and Systematics*, 24, 217-242. <http://dx.doi.org/10.1146/annurev.ecolsys.24.1.217>
- Excoffier, L., Smouse, P. E., & Quattro, J. M. (1992). Analysis of Molecular Variance Inferred from Metric Distances among DNA Haplotypes - Application to Human Mitochondrial-DNA Restriction Data. *Genetics*, 131(2), 479-491.
- Felsenstein, J. (1985). Phylogenies and the Comparative Method. *American Naturalist*, 125(1), 1-15. <http://dx.doi.org/10.1086/284325>
- Foster, S. A., & Janson, C. H. (1985). The Relationship between Seed Size and Establishment Conditions in Tropical Woody-Plants. *Ecology*, 66(3), 773-780. <http://dx.doi.org/10.2307/1940538>

- Freckleton, R. P., & Harvey, P. H. (2006). Detecting non-Brownian trait evolution in adaptive radiations. *PLoS Biol*, 4(11), e373. <http://dx.doi.org/10.1371/journal.pbio.0040373>
- Goudet, J. (2005). HIERFSTAT, a package for R to compute and test hierarchical F-statistics. *Molecular Ecology Notes*, 5(1), 184-186. <http://dx.doi.org/10.1111/j.1471-8278.2004.00828.x>
- Goudet, J., & Buchi, L. (2006). The effects of dominance, regular inbreeding and sampling design on Q(ST), an estimator of population differentiation for quantitative traits. *Genetics*, 172(2), 1337-1347. <http://dx.doi.org/10.1534/genetics.105.050583>
- Gravuer, K., von Wettberg, E., & Schmitt, J. (2005). Population differentiation and genetic variation inform translocation decisions for *Liatris scariosa* var. *novae-angliae*, a rare New England grassland perennial. *Biological Conservation*, 124(2), 155-167. <http://dx.doi.org/10.1016/j.biocon.2005.01.021>
- Grubb, P. J., & Metcalfe, D. J. (1996). Adaptation and inertia in the Australian tropical lowland rain-forest flora: Contradictory trends in intergeneric and intrageneric comparisons of seed size in relation to light demand. *Functional Ecology*, 10(4), 512-520. <http://dx.doi.org/10.2307/2389944>
- Hama, E. (2002). *The wild Violets of Japan in Color*. Tokyo: Seibundo-shinkasha.
- Hedrick, P. W. (1980). Hitchhiking: a comparison of linkage and partial selfing. *Genetics*, 94(3), 791-808.
- Hendry, A. P. (2002). Q(ST) >= not equal < F-ST? *Trends in Ecology & Evolution*, 17(11), 502-502. [http://dx.doi.org/10.1016/S0169-5347\(02\)02603-4](http://dx.doi.org/10.1016/S0169-5347(02)02603-4)
- Hewitt, N. (1998). Seed size and shade-tolerance: a comparative analysis of North American temperate trees. *Oecologia*, 114(3), 432-440. <http://dx.doi.org/10.1007/s004420050467>
- Igari, M. (1996). *Violets of Japan (Nihon-no-sumire)*. Tokyo: Yama-to-keikoku-sha.
- Jimenez-Ambriz, G., Petit, C., Bourrie, I., Dubois, S., Olivieri, I., & Ronce, O. (2007). Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytol*, 173(1), 199-215. <http://dx.doi.org/10.1111/j.1469-8137.2006.01923.x>
- Jorgensen, T. H., Richardson, D. S., & Andersson, S. (2006). Comparative analyses of population structure in two subspecies of *Nigella degenii*: evidence for diversifying selection on pollen-color dimorphisms. *Evolution*, 60(3), 518-528. <http://dx.doi.org/10.1554/05-393.1>
- Lascoux, M., Thorsen, J., & Gullberg, U. (1996). Population structure of a riparian willow species, *Salix viminalis* L. *Genetical Research*, 68(1), 45-54. <http://dx.doi.org/10.1017/S0016672300033875>
- Lecorff, J. (1993). Effects of Light and Nutrient Availability on Chasmogamy and Cleistogamy in an Understory Tropical Herb, *Calathea-Micans* (Marantaceae). *American Journal of Botany*, 80(12), 1392-1399. <http://dx.doi.org/10.2307/2445667>
- Leinonen, T., O'Hara, R. B., Cano, J. M., & Merila, J. (2008). Comparative studies of quantitative trait and neutral marker divergence: a meta-analysis. *J Evol Biol*, 21(1), 1-17. <http://dx.doi.org/10.1111/j.1420-9101.2007.01445.x>
- Levin, D. A. (2000). *The Origin, Expansion, and Demise of Plant Species*. New York: Oxford University Press.
- Masuda, M., & Yahara, T. (1994). Reproductive Ecology of a Cleistogamous Annual, *Impatiens-Noli-Tangere* L., Occurring under Different Environmental-Conditions. *Ecological Research*, 9(1), 67-75. <http://dx.doi.org/10.1007/BF02347243>
- Masuda, M., Yahara, T., & Maki, M. (2004). Evolution of floral dimorphism in a cleistogamous annual, *Impatiens noli-tangere* L. occurring under different environmental conditions. *Ecological Research*, 19(6), 571-580. <http://dx.doi.org/10.1111/j.1440-1703.2004.00673.x>
- Mattila, T., & Salonen, V. (1995). Reproduction of *Viola mirabilis* in relation to light and nutrient availability. *Canadian Journal of Botany-Revue Canadienne De Botanique*, 73(12), 1917-1924.
- Mayr, E. (1963). *Animal Species and Evolution*. Cambridge, MA: Harvard University press.
- McKay, J. K., & Latta, R. G. (2002). Adaptive population divergence: markers, QTL and traits. *Trends in Ecology & Evolution*, 17(6), 285-291. [http://dx.doi.org/10.1016/S0169-5347\(02\)02478-3](http://dx.doi.org/10.1016/S0169-5347(02)02478-3)
- Merilä, J., & Crnokrak, P. (2001). Comparison of genetic differentiation at marker loci and quantitative traits. *Journal of Evolutionary Biology*, 14(6), 892-903. <http://dx.doi.org/10.1046/j.1420-9101.2001.00348.x>

- Morjan, C. L., & Rieseberg, L. H. (2004). How species evolve collectively: implications of gene flow and selection for the spread of advantageous alleles. *Molecular Ecology*, 13(6), 1341-1356. <http://dx.doi.org/10.1111/j.1365-294X.2004.02164.x>
- Navarro, C., Cavers, S., Pappinen, A., Tigerstedt, P., Lowe, A., & Merila, J. (2005). Contrasting quantitative traits and neutral genetic markers for genetic resource assessment of Mesoamerican *Cedrela odorata*. *Silvae Genetica*, 54(6), 281-292.
- Petit, C., Freville, H., Mignot, A., Colas, B., Riba, M., Imbert, E., ... Olivieri, I. (2001). Gene flow and local adaptation in two endemic plant species. *Biological Conservation*, 100(1), 21-34. [http://dx.doi.org/10.1016/S0006-3207\(00\)00204-4](http://dx.doi.org/10.1016/S0006-3207(00)00204-4)
- R Development Core Team (2006). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>.
- Raeymaekers, J. A. M., Van Houdt, J. K. J., Larmuseau, M. H. D., Geldof, S., & Volckaert, F. A. M. (2007). Divergent selection as revealed by P-ST and QTL-based F-ST in three-spined stickleback (*Gasterosteus aculeatus*) populations along a coastal-inland gradient. *Molecular Ecology*, 16(4), 891-905. <http://dx.doi.org/10.1111/j.1365-294X.2006.03190.x>
- Reich, P. B., Walters, M. B., & Ellsworth, D. S. (1992). Leaf Life-Span in Relation to Leaf, Plant, and Stand Characteristics among Diverse Ecosystems. *Ecological Monographs*, 62(3), 365-392. <http://dx.doi.org/10.2307/2937116>
- Reich, P. B., Walters, M. B., & Ellsworth, D. S. (1997). From tropics to tundra: global convergence in plant functioning. *Proc Natl Acad Sci U S A*, 94(25), 13730-13734. <http://dx.doi.org/10.1073/pnas.94.25.13730>
- Reich, P. B., Wright, I. J., Cavender-Bares, J., Craine, J. M., Oleksyn, J., Westoby, M., & Walters, M. B. (2003). The evolution of plant functional variation: Traits, spectra, and strategies. *International Journal of Plant Sciences*, 164(3), S143-S164. <http://dx.doi.org/10.1086/374368>
- Rieseberg, L. H., & Burke, J. M. (2001). The biological reality of species: gene flow, selection, and collective evolution. *Taxon*, 50(1), 47-67. <http://dx.doi.org/10.2307/1224511>
- Schemske, D. W. (1978). Evolution of Reproductive Characteristics in *Impatiens* (Balsaminaceae) - Significance of Cleistogamy and Chasmogamy. *Ecology*, 59(3), 596-613. <http://dx.doi.org/10.2307/1936588>
- Schluter, D. (2001). Ecology and the origin of species. *Trends Ecol Evol*, 16(7), 372-380. [http://dx.doi.org/10.1016/S0169-5347\(01\)02198-X](http://dx.doi.org/10.1016/S0169-5347(01)02198-X)
- Schoen, D. J., & Lloyd, D. G. (1984). The Selection of Cleistogamy and Heteromorphic Diaspores. *Biological Journal of the Linnean Society*, 23(4), 303-322. <http://dx.doi.org/10.1111/j.1095-8312.1984.tb00147.x>
- Slatkin, M. (1985). Gene Flow in Natural-Populations. *Annual Review of Ecology and Systematics*, 16, 393-430. <http://dx.doi.org/10.1146/annurev.ecolsys.16.1.393>
- Spitze, K. (1993). Population structure in *Daphnia obtusa*: quantitative genetic and allozymic variation. *Genetics*, 135(2), 367-374.
- Sterck, F. J. (1999). Crown development in tropical rain forest trees in gaps and understorey. *Plant Ecology*, 143(1), 89-98. <http://dx.doi.org/10.1023/A:1009889414418>
- Thibert-Plante, X., & Hendry, A. P. (2011). The consequences of phenotypic plasticity for ecological speciation. *J Evol Biol*, 24(2), 326-342. <http://dx.doi.org/10.1111/j.1420-9101.2010.02169.x>
- Toyama, H., & Yahara, T. (2009). Comparative phylogeography of two closely related *Viola* species occurring in contrasting habitats in the Japanese archipelago. *J Plant Res*, 122(4), 389-401. <http://dx.doi.org/10.1007/s10265-009-0235-7>
- Waldmann, P., & Andersson, S. (1998). Comparison of quantitative genetic variation and allozyme diversity within and between populations of *Scabiosa canescens* and *S-columbaria*. *Heredity*, 81, 79-86. <http://dx.doi.org/10.1046/j.1365-2540.1998.00379.x>
- Whitlock, M. C. (2008). Evolutionary inference from Q(ST). *Molecular Ecology*, 17(8), 1885-1896. <http://dx.doi.org/10.1111/j.1365-294X.2008.03712.x>
- Widén, B., Andersson, S., Rao, G. Y., & Widén, M. (2002). Population divergence of genetic (co)variance matrices in a subdivided plant species, *Brassica cretica*. *Journal of Evolutionary Biology*, 15(6), 961-970. <http://dx.doi.org/10.1046/j.1420-9101.2002.00465.x>

- Wilken, D. H. (1982). The Balance between Chasmogamy and Cleistogamy in *Collomia-Grandiflora* (Polemoniaceae). *American Journal of Botany*, 69(8), 1326-1333. <http://dx.doi.org/10.2307/2442758>
- Wright, I. J., Reich, P. B., Cornelissen, J. H. C., Falster, D. S., Groom, P. K., Hikosaka, K., ... Westoby, M. (2005). Modulation of leaf economic traits and trait relationships by climate. *Global Ecology and Biogeography*, 14(5), 411-421. <http://dx.doi.org/10.1111/j.1466-822x.2005.00172.x>
- Wright, S. (1951). The Genetical Structure of Populations. *Annals of Eugenics*, 15(4), 323-354.