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ORIGINAL ARTICLE

Antagonistic selection factors induce a continuous population divergence in a polymorphism

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Understanding the relative importance of selection and stochastic factors in population divergence of adaptive traits is a classical topic in evolutionary biology. However, it is difficult to separate these factors and detect the effects of selection when two or more contrasting selective factors are simultaneously acting on a single locus. In the damselfly *Ischnura senegalensis*, females exhibit color dimorphism and morph frequencies change geographically. We here evaluated the role of selection and stochastic factors in population divergence of morph frequencies by comparing the divergences in color locus and neutral loci. Comparisons between population pairwise F_{ST} for neutral loci and for the color locus did not detect any stochastic factors affecting color locus. Although comparison between population divergence in color and neutral loci using all populations detected only divergent selection, we detected two antagonistic selective factors acting on the color locus, that is, balancing and divergent selection, when considering geographical distance between populations. Our results suggest that a combination of two antagonistic selective factors, rather than stochastic factors, establishes the geographic cline in morph frequency in this system.

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INTRODUCTION

Understanding mechanisms responsible for establishing spatial patterns in traits or allele frequencies is a central issue in evolutionary biology and ecology (Endler, 1977; Huey *et al.*, 2000; Saccheri *et al.*, 2008). Spatial patterns in selectively neutral genetic variation result from random factors alone. However, spatial variation in adaptive traits, including qualitative and quantitative traits, may be produced by both selection and genetic drift (McKay and Latta, 2002; Strand *et al.*, 2012). Thus, quantifying the relative roles of selection and historical/ongoing stochastic factors is important to understand the process of population divergence in adaptive phenotypic and genotypic traits (Schmidt *et al.*, 2008).

Clines in genotype-specific morph/allele frequency are suitable model systems to study the relative importance of selection and historical/ongoing genetic drift in phenotypic population divergence, because the establishment of clines is potentially affected by both selection and historical/ongoing genetic drift (Endler, 1973; Whibley *et al.*, 2006). The mechanisms that establish clines in morph frequency can be roughly classified into three categories (Endler, 1977). In two of these three mechanisms, divergent selection derived from gene-by-environment (G × E) interactions underlies the establishment of a cline in morph frequency; the fitness advantage of each morph differentially changes across the environmental gradient and reverses across an equilibrium (balancing) point, where each phenotype has equal fitness (Endler, 1973). However, G × E itself leads to creation of a stepwise pattern in morph frequency across an equilibrium point, because in each population, a single morph with the highest fitness in each population should dominate.

Theoretically, a smooth cline in morph frequency has been suggested to be produced by the homogenizing evolutionary forces (Endler, 1977). The first mechanism is based on gene flow. Gene flow among populations makes the cline smooth around the balancing point, indicating that the cooperation of divergent selection derived from G × E and ongoing gene exchange over the equilibrium point may establish a smooth cline in morph frequency. Note that because gene flow occurs among adjacent populations, the clines established by gene flow are expected to be steep (Endler, 1973). The second mechanism is based on balancing selection, because it leads to the coexistence of multiple morphs having different potential fitness within a population in a certain spatial range (Endler, 1973; Takahashi *et al.*, 2011). Thus, the cooperation of divergent and balancing selection on morphs, that is, antagonistic multiselection, may explain clines in morph frequency. Alternatively, according to the third mechanism, clines may be established without divergent and balancing selection. In this case, the clinal patterns in morph frequency are observed at hybrid zones, which arise from secondary contact between two historically allopatric populations with different alleles (Latta and Mitton, 1999; Whibley *et al.*, 2006). Thus, the combination of two types of genetic drift, that is, secondary contact and gene flow between adjacent populations, results in a smooth cline in morph frequency. When this mechanism is occurring, morph frequencies do not always correlate with environmental factors, unlike in the first two mechanisms.

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Smooth, large-scale clines in morph frequency have been reported for many animals and plants (Komai *et al.*, 1950; Dearn, 1981; Hodgins and Barrett, 2008). Latitudinal and altitudinal clines in body color polymorphisms are known from various species of damselflies, where frequencies of female color morphs change geographically (Iserbyt *et al.*, 2010; Sanchéz-Guillén *et al.*, 2011; Takahashi *et al.*, 2011). Some of these clines appear to be difficult to explain by the combination of divergent selection and the effects of gene flow, as gene flow in damselflies is generally restricted (Sato *et al.*, 2008; Allen *et al.*, 2010, but see Watts *et al.*, 2010). Instead, at least theoretically, the combination of divergent and balancing selection may be a plausible explanation for the establishment and maintenance of large-scale clines (Endler, 1973). In several polymorphic damselflies, empirical tests have been conducted to detect negative frequency-dependent selection (Svensson *et al.*, 2005; Takahashi *et al.*, 2010).

In the common bluetail damselfly *Ischnura senegalensis*, a smooth latitudinal cline in female morph frequency was observed over a distance of 1000 km in Japan (Takahashi *et al.*, 2011). In this system, morphological and behavioral studies implied that antagonistic multiselection factors (divergent and balancing selection) underlie geographic clines in morph frequency in the wild. However, the contributions of gene flow and historical events to the geographic cline in morph frequency had been ruled out by the previous studies (Takahashi *et al.*, 2011). In addition, the relative roles of the selective factors and genetic drift in establishing the geographic-scale clines have not been determined, as in the case of other organisms exhibiting large-scale cline in morph frequency.

Comparing the population differentiation of neutral loci and loci presumed to be subject to selection is a common method used to detect the effect of selection and random genetic drift on population divergence in natural populations (McKay and Latta, 2002), for instance, by comparing the population pairwise F_{ST} values for neutral loci with those for loci suspected to be subject to selection. Correlation between F_{ST} values for neutral loci with those for the loci in question indicates the significance of the effect of random drift on population divergence (Runemark *et al.*, 2010). In addition, insights into the presence of divergent selection, balancing selection and random drift can be obtained by contrasting the degree of genetic differentiation (e.g., F_{ST}) at a focused locus to the degree of differentiation at neutral markers (Gillespie and Oxford, 1998); divergent selection, balancing selection and no selection is acting if ratio F_{ST}(focused)/F_{ST}(neutral) is greater than, smaller than and equal to 1, respectively, as in the case of phenotypic traits (Lynch and Walsh, 1998).

Several recent studies on polymorphic damselflies have utilized this approach, and divergent selection (Wong *et al.*, 2003), balancing selection (Andrés *et al.*, 2000) and historical events (Sánchez-Guillén *et al.*, 2011) have all been detected. However, in reality, it is difficult to accurately detect the selective factors responsible when antagonistic selective factors, for example, divergent and balancing selection, are simultaneously acting on a given locus. Because the deviation of F_{ST} (neutral) from F_{ST} (focused locus) is affected by the relative strength of the two antagonistic selective factors acting on the focused locus, which have contrasting effects on population divergence (F_{ST} (focused locus)), factors detected by such comparisons (divergent selection, balancing selection and random drift) should depend on the degree of variation in directional selection, for example, the spatial scale. In practice, previous studies using polymorphic damselflies have reached different conclusions regarding the relative importance of selective factors and drift (Andrés *et al.*, 2000; Wong *et al.*, 2003; Sánchez-Guillén *et al.*, 2011). We here assessed the importance of selective factors and genetic drift in population divergence in the damselfly *I. senegalensis*, taking spatial scale into consideration, and thus revealed the mechanism establishing the cline in morph frequency in this system.

MATERIALS AND METHODS

Study species

Ischnura senegalensis is a non-territorial damselfly that inhabits the open and sunny edges of ponds. Males are monomorphic, whereas females exhibit color dimorphism (an andromorph and a gynomorph) (Takahashi *et al.*, 2010; 2012). The female color morphs are determined by two alleles at a single autosomal locus with female-limited expression (Takahashi, 2011). The allele for the andromorph is recessive to that of the gynomorph, similar to other female dimorphic damselflies (Johnson, 1964). A previous study suggested that morph frequencies show clinal variation along a latitudinal gradient (Takahashi, 2011).

In this system, morphological analysis suggested the presence of divergent selection derived from G × E in a spatially heterogeneous environment, where potential fitness differentially changed with latitude between morphs (Takahashi *et al.*, 2011), and behavioral observations and fitness analysis suggested negative frequency-dependent selection, where rare morphs gain fitness advantages by avoiding male mating harassment (Takahashi and Watanabe, 2009; Takahashi *et al.*, 2010). Mathematical modeling showed that the latitudinal cline in morph frequency of *I. senegalensis* can be explained by the combination of divergent selection and negative frequency-dependent selection (Takahashi *et al.*, 2011).

Morph frequency and molecular sampling

During the spring and early summer months (April–August) of 2009–2011, morph frequencies were recorded using the line census method in 36 local populations (north latitude 23°–38°, Supplementary Table S1). Census lines (10–500 m) were set along the edge of the ponds. Each line census was conducted in the morning by walking slowly along the line in order to avoid disturbance. To estimate morph frequency, we recorded the number of males and the two female morphs found within 1 m of the line, on both sides. The mean number of females detected ± s.e in each line census was 56.35 ± 11.31. The location and sampling date are given in Figure 1a and Supplementary Table S1. Adults were collected during their first generation, which had overwintered as larvae. For the Amami population alone, morph frequency was estimated by 30 min of random sampling along the water's edge. For all local populations, latitude and longitude were determined using Google Earth.

For the molecular experiments, adult individuals of *I. senegalensis* were collected from 29 of the 36 populations for which morph frequencies were determined. The thorax of each captured individual was immediately preserved in 2 ml absolute ethanol and stored at $-20\,^{\circ}$C until DNA extraction. Both males and females were used in genetic analyses. The average number of individuals used for microsatellite analysis in each population was 18.7 ± 5.71 (± s.d.) (see Supplementary Table S1 for details on the number of specimens examined).

Environmental factors

To examine the relationship between climatic factors and morph frequency, climate data from 2008 (annual mean of precipitation, temperature and solar radiation) at the 1-km mesh level were collected from the database gamsDB (http://www.agrienv.dc.affrc.go.jp/integrated_db/web/index.html). Principal component analysis was conducted using the average of the monthly averages for climate data in each population, and the correlation between principal components and morph frequency was analyzed by Pearson's correlation test.

To detect the critical temperature for morph frequency, the relationships between mean monthly temperatures and morph frequency over 12 months were examined using a generalized additive model (GAM). Relationships between the mean monthly temperature for all populations and the *P*-value or r^2 derived from GAM (12 data points for each) were analyzed.

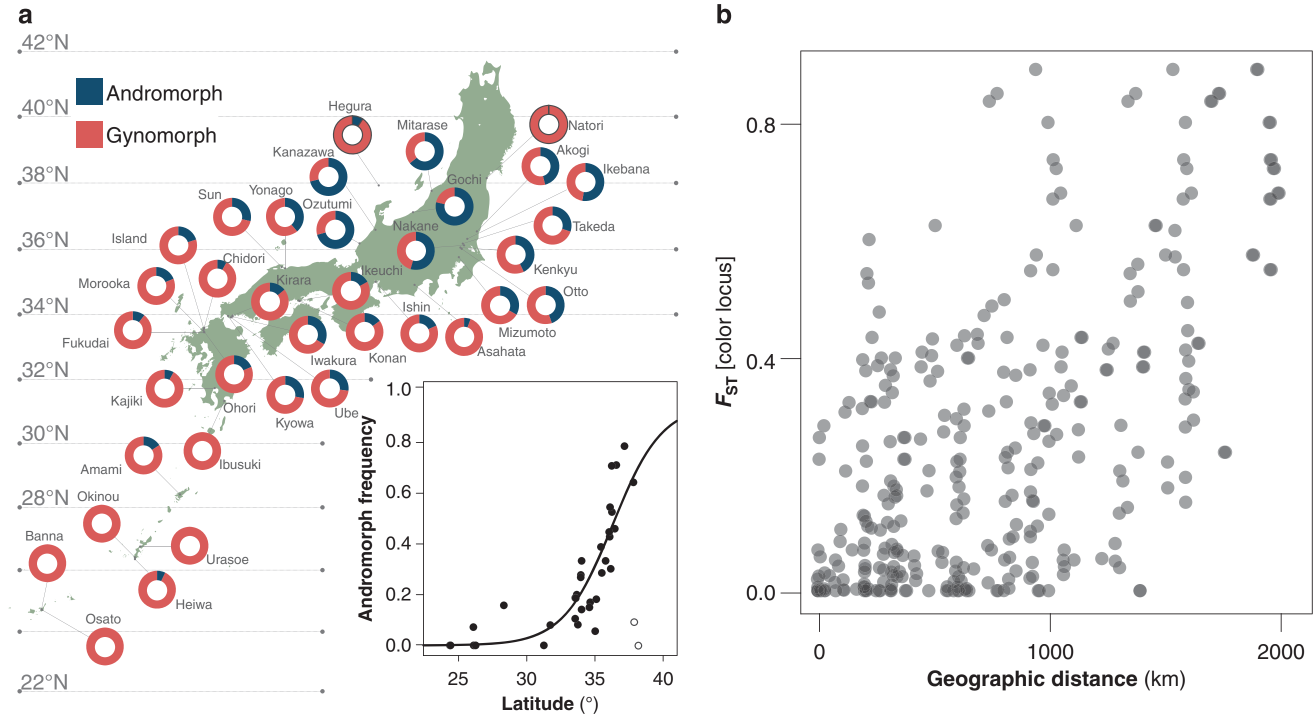

Figure 1 Geographic variation in morph frequency in females of the common bluetail *Ischnura senegalensis*. (**a**) Morph frequencies for each local population are shown as a pie chart (blue: andromorph; red: gynomorph). The frequency of andromorphs increased with latitude, except in the two northern populations (pie charts with thick black lines). The inset Figure shows logistic regression with the latitude, excluding the two northernmost populations (open circles). (**b**) Relationship between pairwise geographic distances and pairwise genetic distances (F_{ST}) for the color locus, representing isolation-by-distance.

DNA extraction and genotyping

The thorax muscles of individual damselflies were incubated for 3 h in extraction buffer (0.1 M Tris pH 8.0, 10 mM EDTA and 0.2 mg ml^{-1} proteinase K) at 50 °C, and total genomic DNA was purified using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA).

Alleles at six previously developed microsatellite loci (I002, I015, I053, I058, I034 and I210) (Wellenreuther *et al*., 2010) were amplified by PCR. The PCR products were separated and alleles were detected using an ABI 3130 sequencer. GeneMapper 3.0 (Applied Biosystems, Foster City, CA, USA) was used to determine the genotypes of the individuals for the neutral loci. Loci were tested for the Hardy–Weinberg equilibrium and linkage disequilibrium using the program GENEPOP version 3.4 (Raymond and Rousset, 1995) operating a Markov chain approximation with 100 000 interactions with 1000 steps. Bonferroni corrections were performed on the probability values for each test. One microsatellite locus (I216) showed signs of selection (LOSITAN software, Beaumont and Nichols, 1996), probably due to frequent bottleneck events, which was suggested by the population genetic analysis based on mitochondrial DNA (see Supplementary Table S2 and Supplementary Figure S1). As this locus was suggested to be neutral in a closely related species, *I. elegans* (Wellenreuther *et al*., 2010), we used all six microsatellite loci for our analysis.

Genotype frequencies at the color locus in each population were estimated on the basis of the phenotypic frequency data from the wild populations. The inheritance system for the two female color morphs has been reported for *I. senegalensis*; the two morphs are controlled by two alleles at a single autosomal locus with sex-limited expression (Takahashi, 2011). The andromorphic (*d*) allele is recessive to the gynomorphic (*D*) allele, as in the case of other female dimorphic damselflies (Johnson, 1964). Allelic frequencies in each population were estimated on the basis of color morph frequencies, assuming Hardy–Weinberg equilibrium within populations, and then the allele frequencies were used to estimate genotypic frequencies of *DD*, *Dd* and *dd*, assuming random mating.

Characterizing population genetic structure

The program STRUCTURE version 2.3 (Prichard *et al*., 2000) was run on individual multilocus genotypes for a number of clusters, *K*, with a burn-in of 100 000 interactions and a run of 1 000 000 iterations (five replicates at each of $K=1$–28). The optimal number of clusters, *K*, was decided according to the *ad hoc* statistic Δ*K* based on the rate of change in the log probability of data between successive *K* values (Evanno *et al*., 2005).

Population pairwise F_{ST} values based on the genotype frequencies of the microsatellite loci, and the color locus for all possible population combinations were calculated using the program package Arlequin 3.5 (Excoffier *et al*., 2005). To test the pattern of isolation-by-distance, relationships between these two F_{ST} values and population pairwise geographic distance (simple Euclidean distance) were analyzed.

Detection of genetic drift and selective factors

We tested for a correlation between the F_{ST} values for microsatellite loci and the putatively selected locus (color locus). The degree of correlation can inform us about the importance of genetic drift and selection in maintaining color morph frequencies (Runemark *et al*., 2010). A strong correlation would suggest that the color morph frequency in local populations is largely affected by genetic drift, whereas a weak correlation would be indicative of selection (Sánchez-Guillén *et al*., 2011).

To compare population divergence in color and neutral loci, we first tested for a deviation of F_{ST} values for the color locus from F_{ST} values for all microsatellite loci. In general, F_{ST} for neutral loci is affected by the mutation rate at each locus (Edelaar and Björklund, 2011), and comparison between F_{ST} values for the color locus and F_{ST} values for multiple microsatellite loci is important to estimate the random expectation of population divergence. As mentioned above, because the relative importance of divergent and balancing selection may be affected by the spatial scale, the effect of one selective factor may be masked by the effect of another selective factor at a particular geographic scale. Thus, in the present study, population pairwise F_{ST} (color) and population pairwise F_{ST} (neutral) were classified into four classes on the basis of geographic distance: 0–10, 10–100, 100–1000 and >1000 km. To compare the relative size of F_{ST} (color) to F_{ST} (neutral) along geographic classes, F_{ST} (color)/F_{ST} (neutral) (that is, standardized F_{ST} (color)) was calculated for all population pairs, and the deviations of the mean of F_{ST}

(color)/F_{ST} (neutral) from 1 were analyzed in each geographic category; divergent selection, balancing selection is suggested if ratio F_{ST} (focused)/F_{ST} (neutral) is greater than and smaller than 1, respectively (Lynch and Walsh, 1998). On the other hand, F_{ST} (color)/F_{ST} (neutral), which does not differ from 1 generally suggests no selection on focused locus, but also suggests that the effect of divergent selection counterbalances that of balancing selection.

Statistical analyses

Statistical analyses were performed using R version 2.14.0 (R Development Core Team, 2011). To analyze the overall pattern of geographic variation in morph frequency, two northern populations where few or no andromorphs were found were excluded from our analyses. One of the two populations (Natori) was recently created, and it was suspected that individuals carrying the andromorphic allele had not been introduced to date (that is, founder effect) (Takahashi *et al.*, 2011). Another population (Hegura, 38°N, Figure 1a and Supplementary Table S1) is located on a northern island, where the population is established in a hot spring pond, in which the water temperature is high throughout the year.

The relationship between mean monthly temperature and log-transformed *P*-values derived from GAM and between mean monthly temperature and r^2 derived from GAM were analyzed using Pearson's correlation test. We tested for a possible pattern of isolation-by-distance between all population pairs ($N=29$). We also applied a Mantel test to investigate whether the pairwise matrix of genetic differentiation F_{ST} is correlated with the matrix of geographic distances. The Mantel test was performed using the program PASSaGE (Rosenberg and Anderson, 2011). The correlation between F_{ST} (color) and F_{ST} (neutral) was also tested using a Mantel test. As F_{ST} values for microsatellite loci were weakly but significantly correlated with geographic distance, a partial Mantel test with geographic distance as the covariate was used to detect correlation between F_{ST} for neutral and color loci. As F_{ST} values are calculated in a pairwise manner, they are not independent. Therefore, the mean and confidence interval of F_{ST} (color)/F_{ST} (neutral) was estimated by resampling procedure (permutation and bootstrapping). Statistically significant deviation from 1 was identified if the 95% confidence intervals did not cross 1.

RESULTS

Cline in morph frequency

A smooth latitudinal cline was observed in female morph frequency in Japan (Figure 1a). The frequency of andromorphs in each local population ranged from 0% (South) to 79% (North). Logistic regression analysis showed that the frequency of andromorphs increased with latitude ($t=5.077$, $P<0.001$), becoming 50% at the northern latitude of 36 degrees (inset in Figure 1a). In southern populations on Ishigaki Island (including Osato and Banna, 25°N) and Okinawa Island (including Okinou and Urasoe, 27°N), no andromorphs were detected in the line census because of the small sample size; however, we have detected a small number of andromorphs on these islands, indicating that the cline is wider than 2000 km (23–37°N).

A significant correlation was found between geographic distance and the pairwise F_{ST} for the color locus (Mantel test, $P<0.001$), indicating that adjacent populations showed similar morph frequencies, but distantly positioned populations showed different morph frequencies (Figure 1b).

Environmental factors

Principal component analysis detected two effective dimensions (PC1 and PC2) that explained 89.4% of the total variance. PC1 summarized temperature and solar radiation, and PC2 mainly summarized precipitation. Morph frequency significantly correlated with PC1 (index of warmth), although no significant correlation was found between PC2 (index of weather) and morph frequency (Figure 2a and b), suggesting that morph frequency was determined by temperature rather than weather conditions.

GAM analyses showed that morph frequency significantly correlated with each month's temperature ($P<0.05$ for all 12 months). The *P*-value was lower in low-temperature months than in high-temperature months (Figure 2c). Likewise, r^2 was higher in winter than in summer (Figure 2d). These consistently indicate that temperature during the larval stages (particularly January and February) explain the cline in morph frequency.

Genetic structure based on microsatellite loci

All microsatellite loci were polymorphic with 2–19 alleles within a single population (see Supplementary Tables S3 and S4 for details).

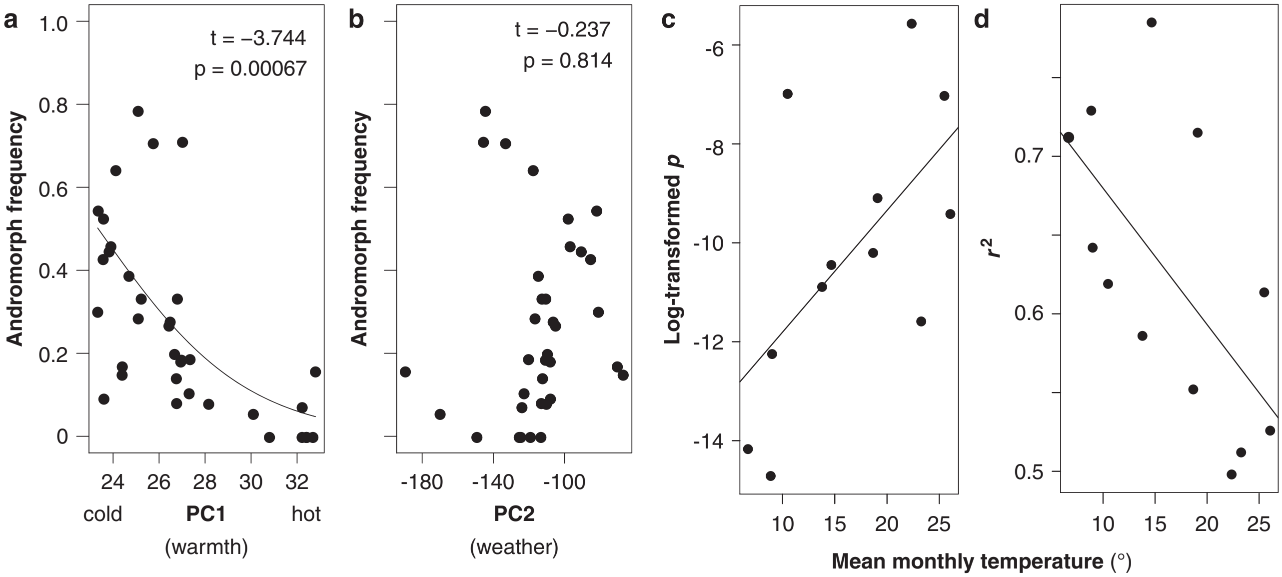

Figure 2 Relationship between climatic factors and morph frequency. Morph frequency was significantly correlated with PC1 (**a**) but not with PC2 (**b**). *P*-values and r^2 based on 12 GAM regressions using the monthly temperature and morph frequency of 28 populations showed positive ($t=2.399$, $P<0.037$) and negative ($t=-2.543$, $P<0.029$) correlations with mean monthly temperature of all populations (**c**, **d**), respectively, suggesting temperature in colder months explained the observed variation in morph frequency.

STRUCTURE analysis indicated that the number of clusters should be three in this species (Supplementary Figure S2). No clinal pattern along a latitudinal gradient was found, and some adjacent populations were classified into different genetic clusters (Figure 3), suggesting a low rate of migration among populations. The Mantel test detected an isolation-by-distance pattern ($P = 0.022$), but the pattern was not clear (inset in Figure 3). Pairwise F_{ST} for the neutral maker seems to be independent of geographic distance, as suggested by analysis based on mitochondrial DNA (Supplementary Figure S1).

Detection of effects of genetic drift

The Mantel test detected a significant correlation between the degree of differentiation in the set of microsatellite loci and that based on the color locus ($P = 0.019$), but the pattern was not clear (Figure 4). Indeed, the partial Mantel test suggests that the degree of differentiation in the set of microsatellite loci did not correlate with that based on the color locus ($P = 0.209$), indicating that strong correlations did not exist between F_{ST} for neutral markers and color locus. No correlation was also found between the degree of differentiation (F_{ST}) based on mitochondrial DNA haplotypes and that based on the color locus (Supplementary Figure S3). These results suggested that the geographic divergence in morph frequency cannot be explained by genetic drift.

Detection of selective factors

When all population pairs were included in the analysis, the F_{ST} (color) value was larger than the F_{ST} (neutral) value based on all six microsatellite loci (neutral: 0.104 (confidence interval: 0.095–0.113), color: 0.256 (confidence interval: 0.230–0.282)), suggesting that divergent selection predominantly determined the overall geographic pattern of alleles at the color locus in Japan. However, F_{ST} (color)/F_{ST} (neutral) for nearby population pairs (0–10 km apart) was smaller than 1 (Figure 5). This suggests that balancing selection predominantly acted on the color locus at a small geographic scale. On the other hand, F_{ST} (color)/F_{ST} (neutral) based on distant populations (100–1000 km and >1000 km) was significantly >1, suggesting that divergent selection primarily governed the geographic divergence in the color locus at a large geographic scale. At the intermediate geographic scale (10–100 km), F_{ST} (color)/F_{ST} (neutral) did not significantly deviate from 1. F_{ST} (color)/F_{ST} (neutral) along geographic class were varied among six microsatellite loci, but the value based on each loci showed similar pattern to average neutral explanation based on all loci (Supplementary Figure S4).

DISCUSSION

Geographic variation among populations in adaptive traits provides key insights into the relative roles of selection and drift in population divergence and speciation (McKay and Latta, 2002; Iserbyt *et al*., 2010). Clinal variation in morph (allele) frequency could be established by the combination of secondary contact and gene flow (Whibley *et al*., 2006), the combination of divergent selection and gene flow, the combination of divergent and balancing selection (Endler, 1977) or a complex combination of these three factors. In the

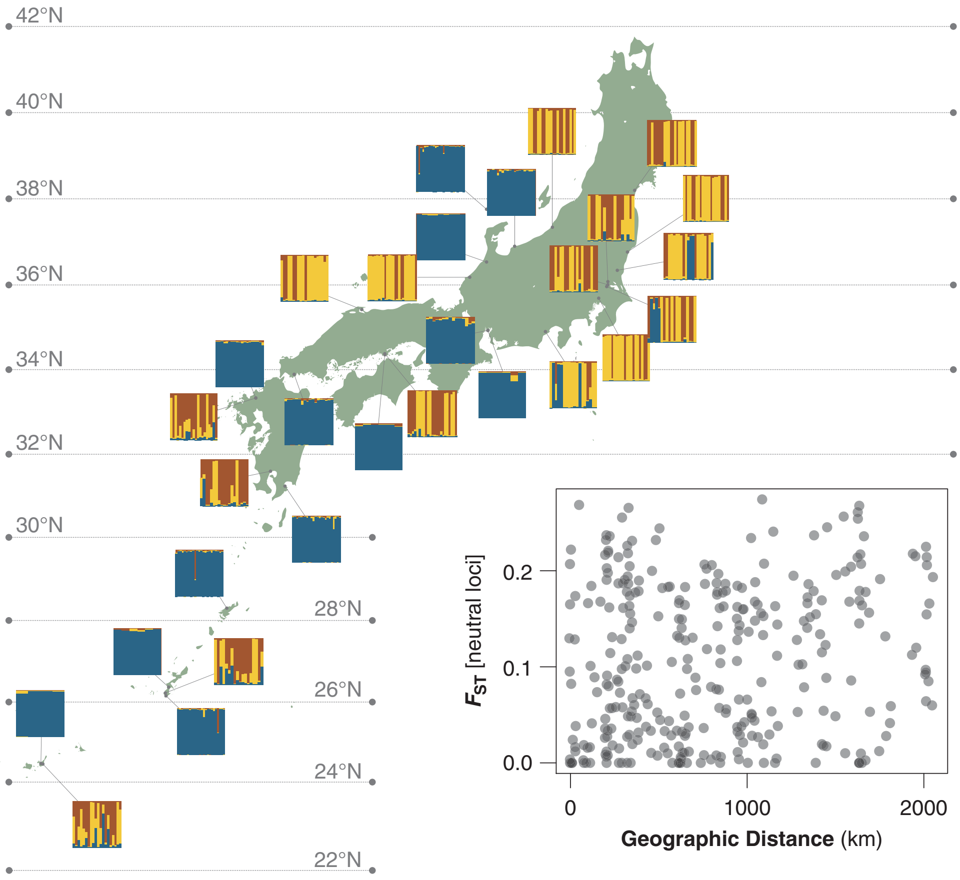

Figure 3 Genetic structure based on six microsatellite loci. Estimated population structure of *I. senegalensis* from Bayesian structure analyses using the program STRUCTURE. Individual Bayesian assignment probabilities for $K = 3$ (see supplementary Figure S2 for detail). Individuals are represented by thin vertical lines, which are partitioned into K segments representing each individual's estimated membership fraction. The inset figure shows no significant relationship between pairwise geographic distances and pairwise genetic distances (F_{ST}) for the microsatellite loci.

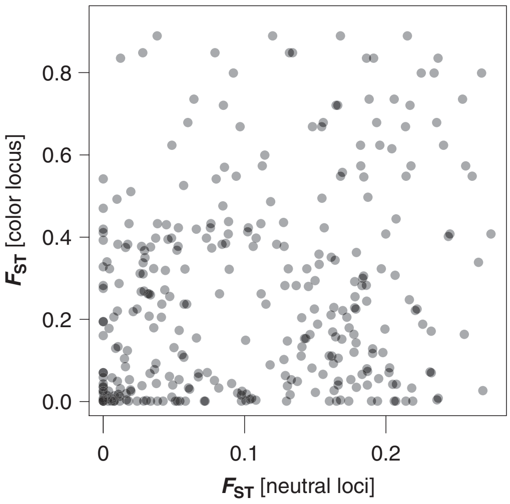

Figure 4 Correlation between the pairwise genetic distances (F_{ST}) for color loci and microsatellite loci. No significant relationship was observed between color morph divergence and neutral divergences.

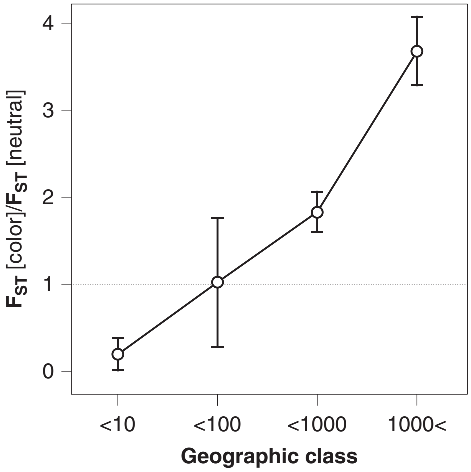

Figure 5 Deviation of color divergence from neutral divergence depending on the geographic scale (0–10, 10–100, 100–1000 and >1000 km) based on all microsatellite loci. Because geographic distances of population pairs are biased, that is, there were few pairs located in close proximity, population pairwise F_{ST} (color) and population pairwise F_{ST} (neutral) were analyzed under four discontinuous classes instead of using geographic distance as a continuous variable. Mean F_{ST} (color)/F_{ST} (neutral) based on six microsatellite loci was significantly smaller in the small geographic class (0–10 km apart), and larger in the larger class (100–1000 km and >1000 km), it was significantly >1.

present study, balancing and divergent selection were detected by comparison of F_{ST} for neutral and selected loci, although analyses of isolation-by-distance failed to detect evidence for strong gene flow and historical secondary contact. This indicates that a combination of divergent and balancing selection, that is, antagonistic multiselective factors, contributes to the establishment of clinal variation in morph frequency in this system. The clear correlation between temperature and morph frequency supports the hypothesis and suggests that temperature affects the cline in morph frequency more strongly than other environmental factors.

Although the deviation of F_{ST} (color) from F_{ST} (neutral) varied among loci probably because of the differences in the evolutionary rate and current allele richness among the six loci, comparisons between F_{ST} (color) and F_{ST} (neutral) over the six loci consistently suggested that balancing selection is predominantly acting on the color locus at a small geographic scale and divergent selection predominates at a large geographic scale. Note that, population differentiation is generally estimated to be smaller at highly variable loci (for example, microsatellite loci) than at less variable loci (for example, color locus) (Hedrick, 1999), suggesting overestimation of F_{ST} (color)/F_{ST} (neutral) in the present study. This indicates that balancing selection is acting on the color loci more strongly than we estimated. In actual, balancing selection may be predominantly acting on color locus at two smaller geographic scales, 0–10 and 10–100 km. Anyway, our results suggest that both balancing and divergent selection are acting on color locus, and their relative strength change with geographic scale among populations.

Geographic genetic structure

Dispersal is generally limited to a small geographic scale in damselflies (Sato *et al.*, 2008; Allen *et al.*, 2010, but see Watts *et al.*, 2010). The present study and previous molecular work have also suggested that local populations tend to be isolated (Watts *et al.*, 2004). However, because of their small body size, adult insects are often carried over long distances across the sea by strong winds, such as typhoons (Corbet, 1999). In the present study, no clear isolation-by-distance patterns were found. An unclear geographic structure has also been reported in another species of the damselfly *Ischnura* (Sanchéz-Guillén *et al.*, 2011). Such geographic genetic structure may result from infrequent, long-distance foundation events and limited gene flow among adjacent populations. Because such non-clinal genetic structure did not coincide with the clinal geographic structure for the color locus, we subsequently confirmed that genetic drift cannot explain the population divergence at the color locus in the current system (cf. Strand *et al.*, 2012).

Mechanisms of divergent selection

In *I. senegalensis*, the geographic pattern of potential fitness estimated on the basis of body size (*cf.* Thompson *et al.*, 2011) showed different patterns for the two morphs, suggesting G × E (Takahashi *et al.*, 2011). The potential fitness of gynomorphs was higher in the south and lower in the north than that of andromorphs, with intersection (where the two morphs show similar potential fitness) at the northern latitude of ~36°N. Such G × E could lead to spatial variation in the strength and direction of selection, that is, divergent selection. In the present study, the equilibrium point (1:1 ratio) in the cline in morph frequency (36°N) coincided with that estimated from fitness data (Takahashi *et al.*, 2011), suggesting that divergent selection determined by potential fitness results in spatial variation in morph frequency.

The strong correlation between morph frequency and temperature in winter, that is, during the larval stage of *I. senegalensis*, suggests that factors relating to larval development lead to differential body size and then divergent selection acting on the color locus. Because adult body size, which correlates with female potential fitness, is determined by development during the larval stage, environmental conditions during the larval stage are expected to lead to morph-specific larval development. Moreover, in the northernmost island population (Hegura Island), where the water temperature is high throughout the year because of the presence of hot springs and the air temperature is always low, showed a lower frequency of andromorphs, indicating that morph frequency is affected by water temperature during the larval developmental stage. This circumstantial evidence suggests that a differential developmental response to temperature correlated with color morph may underlie divergent selection during the adult stage. Although any adaptive significance or developmental constraints leading to a morph-specific response to water temperature remains unclear, a larval developmental response to temperature rather than a morph-specific niche utilization of larvae and adults may contribute to divergent selection.

Mechanism maintaining cline in morph frequency

Theoretically, divergent selection based on G × E itself creates a steep cline (stepwise pattern) across an equilibrium point in the absence of other evolutionary forces antagonistic to local directional selection (Endler, 1977). A smooth cline in morph frequency can be established by gene flow among populations or balancing selection within a population, and the width of the cline is determined by the migration rate or the strength of balancing selection (Takahashi *et al*., 2011). However, as gene flow is highly effective at relatively small scales in these species, some large-scale clines appear to be difficult to explain by the combination of divergent selection and the effects of gene flow. In practice, a cline induced by gene flow is generally steep (Whibley *et al*., 2006; Saccheri *et al*., 2008). Instead, balancing selection, which can maintain genetic polymorphism, has been suggested to induce a large-scale cline in morph frequency (Endler, 1973). In the plant species *Narcissus triandrus*, negative frequency-dependent selection was attributed to a large-scale cline in flower morph frequency (Hodgins and Barrett, 2008). The allele frequency of sickle cell hemoglobin, which may be under balancing selection, also showed a large-scale cline (Piel *et al*., 2010).

As mentioned above, most coenagrionid damselflies are extremely sedentary, with dispersal limited to the area of contiguous habitat, and their lifetime movement has been reported to be restricted to a small area (Watts *et al*., 2004; Sato *et al*., 2008; Allen *et al*., 2010, but see Watts *et al*., 2010). The present study also suggested a low dispersal (migration) rate. Therefore, it is unlikely that gene flow alone causes the smooth geographic clines observed over 2000 km in this system. Indeed, we did not find any effects of genetic drift and gene flow on morph frequency variation. However, in the present study, we detected balancing selection acting on the color locus in *I. senegalensis*, where strong negative frequency-dependent selection within a population has been demonstrated on the basis of behavioral observations and fitness measurements (Takahashi and Watanabe, 2009; Takahashi *et al*., 2010). These results indicate that it is reasonable that the width and position of the current large-scale geographic cline in morph frequency is explained by antagonistic multiselective pressure on a color locus, that is, the combination of divergent selection at the large geographic scale and balancing selection in each population. To the best of our knowledge, we are the first to detect that both balancing selection and divergent selection act on a focused locus in an organism with a large-scale geographic cline in morph frequency in natural populations.

Strategy for detecting antagonistic selective factors

In most genetically polymorphic systems, morph frequencies are relatively stable in local populations but often vary spatially, suggesting that balancing and divergent selection more or less act on polymorphic loci. This means that both divergent and balancing selection should be detected in each system. However, several recent studies on polymorphic damselflies have detected only one of the two selective factors (Andrés *et al*., 2000; Wong *et al*., 2003; Abbott *et al*., 2008; Sánchez-Guillén *et al*., 2011), probably because the effect of a major selective force on population divergence masks the effect of a minor one. In the present study, existing methods (simple comparison between F_{ST}(neutral) and F_{ST}(color) for all population pairs) indeed failed to detect balancing selection but did detect two antagonistic selective factors (balancing and divergent selection) when geographic distance was considered.

Theoretically, balancing selection tends to be detected in populations located within a small geographic area, where the effect of divergent selection is modest, and divergent selection tends to be detected in populations scattered over a large geographic scale, where the small effect of balancing selection is masked by the effect of divergent selection. In addition, the effect of divergent selection on pairwise F_{ST} values may counterbalance that of balancing selection at a given geographic scale. We indeed detected balancing selection using geographically close populations, and divergent selection using geographically distant populations. However, we did not detect any deviation of divergence in the color locus from the neutral expectation at the intermediate spatial scale (10–100 km). Therefore, when a pair of contrasting selective factors may be simultaneously acting on focused loci, we need to consider the spatial scale to accurately detect the selective factors and to avoid under- or overestimation of the effects of genetic drift.

Although in the present study, we approximately used current Euclidean distance as an index of inter-population distance, factors other than current geographic distance, such as the geographic distance over evolutionary time and the presence of mountains, rivers and oceans, may have affected individual movement. If these factors strongly affect genetic distance estimated using neutral genes, an alternative index, for example, resistance distance, should be used for distance-by-distance analyses for F_{ST} (color)/F_{ST} (neutral) (McRea, 2006).

DATA ARCHIVING

Data deposited in the Dryad repository: doi:10.5061/dryad.08h3j.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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