Local ecological divergence of two closely related stag beetles based on genetic, morphological, and environmental analyses

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Abstract
The process of phenotypic adaptation to the environments is widely recognized. However, comprehensive studies integrating phylogenetic, phenotypic, and ecological approaches to assess this process are scarce. Our study aims to assess whether local adaptation may explain intraspecific differentiation by quantifying multidimensional differences among populations in closely related lucanid species, *Platycerus delicatulus* and *Platycerus kawadai*, which are endemic saproxylic beetles in Japan. First, we determined intraspecific analysis units based on nuclear and mitochondrial gene analyses of *Platycerus delicatulus* and *Platycerus kawadai* under sympatric and allopatric conditions. Then, we compared differences in morphology and environmental niche between populations (analysis units) within species. We examined the relationship between morphology and environmental niche via geographic distance. *P. kawadai* was subdivided into the “No introgression” and “Introgression” populations based on mitochondrial COI gene – nuclear ITS region discordance. *P. delicatulus* was subdivided into “Allopatric” and “Sympatric” populations. Body length differed significantly among the populations of each species. For *P. delicatulus*, character displacement was suggested. For *P. kawadai*, the morphological difference was likely caused by geographic distance or genetic divergence rather than environmental differences. The finding showed that the observed mitochondrial–nuclear discordance is likely due to historical mitochondrial introgression following a range of expansion. Our results show that morphological variation among populations of *P. delicatulus* and *P. kawadai* reflects an ecological adaptation process based on interspecific interactions, geographic distance, or genetic divergence. Our results will deepen understanding of ecological specialization processes across the distribution and adaptation of species in natural systems.

Keywords
character displacement, environmental niche, intraspecific variation, mitochondrial introgression, *Platycerus delicatulus*, *Platycerus kawadai*

Taxonomy Classification
Evolutionary ecology
1 | INTRODUCTION

How and why the diversity of life on earth increased over time are key research questions in ecology and biogeography (Bianquart et al., 2013; Cox et al., 2016; Futuyma & Antonovics, 1992; Savolainen et al., 2013; Thomas et al., 2016). Genetic and ecological speciation can occur in different parts of an ancestral species’ range in which contrasting environmental conditions lead directly or indirectly to the evolution of reproductive isolation (Faulkes et al., 2004; Rundle & Nosil, 2005; Schluter, 2001). However, genetic divergence within and among species does not always cause divergence of morphological and other phenotypic traits due to silent mutations and phenotypic convergence (Fitch, 1970; Ujvari et al., 2015). Adaptative phenotypic variation often occurs via the evolution of eco-morphological innovations that contribute to ecological specialization in response to environmental variations or interspecific interactions (Deviector et al., 2010; Mammola et al., 2020). Therefore, evaluation of the phylogenetic constraints on traits and trait–environment relationships can elucidate the mechanisms underlying evolutionary selection and their impact on current ecological patterns.

Phenotypic adaptation among environments is recognized in a wide variety of taxonomic groups (Benito Garzón et al., 2011; Ghalambor et al., 2007; Pavlek & Mammola, 2021; Xue et al., 2019). Considering adaptation via multivariate genetic and trait analyses is essential in such situations. However, comprehensive studies integrating phylogenetic, phenotypic, and ecological approaches to assessing speciation process and identifying phenotypic variations correlated with local adaptation have usually been neglected.

Here, we investigated inter- and intraspecific relationships using genetic, morphological, and ecological data for two closely related Platycerus beetles, Platycerus delicatulus Lewis, 1883, and Platycerus kawadai Fujita and Ichikawa, 1982, to explore how local adaptation shapes their habitat preference. *P. delicatulus* and *P. kawadai* of the family Lucanidae are endemic to Japan and exhibit geographic genetic variations (Kubota et al., 2011). Both species prefer mature cool temperate deciduous broad-leaved forests. *P. delicatulus* has a wide distribution across the main islands of Japan, except Hokkaido. *P. kawadai* appears to be endemic to central Japan (Figure 1). Both species co-occur throughout the range of *P. kawadai*, although some differences in host wood preference have been observed. More specifically, *P. delicatulus* and *P. kawadai* prefer hard and dry decaying wood as their larval resources, whereas all other Platycerus species in Japan prefer soft and wet decaying wood on the forest floor. However, *P. delicatulus* is more abundant at lower elevations, especially on thick decaying wood, and *P. kawadai* tends to target thin decaying wood at higher elevations (Kubota et al., 2020). Two species would lose large portions of present suitable area under climate change (Zhang & Kubota, 2021). Phylogenetically, the two species diverged approximately 1 million years ago, and no hybridization between them has been recorded (Kubota et al., 2011; Zhu et al., 2020). *P. delicatulus* and *P. kawadai* are sister species with similar morphological and ecological attributes, such that sympatric distributions might lead to ecological divergence. Congeneric and ecologically similar species are considered good models for studies of ecological divergence, and thus these two species provide an opportunity to explore mechanisms underlying niche evolution and develop policies for insect management and conservation strategies.

The present study aimed to quantify multidimensional differences among populations that may arise due to local adaptation in the closely related species *P. delicatulus* and *P. kawadai*. First, we estimated the intra- and interspecific evolutionary dynamics of these two species across their ranges and constructed intraspecific analysis units using integrated phylogenetic results for

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**Figure 1** Occurrence records of *Platycerus delicatulus* (a) and *Platycerus kawadai* (b) at the collection sites in Japan.
both species under sympatric or allopatric conditions. We then explored differences in morphology and environmental niche among the populations within each species. We examined the relationship between morphology and environmental niche via geographic distance to assess whether local adaptation may explain population differentiation.

2 | METHODS

2.1 | Molecular procedures and phylogenetic analyses

This study focused on *P. delicatulus* and *P. kawadai* individuals collected from 2005 to 2020 for genetic analysis across the entire geographic range of these two species (Figure 1). The collection sites of the two species are listed in Appendix 1. Besides, *Platycerus akitaiorum* Imura, 2007, and *Platycerus sugitai* Okuda & Fujita, 1987, were used as outgroups.

In this study, we determined 94 and 45 sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene and nuclear internal transcribed spacer (ITS) region, respectively (Appendix 2). Genomic DNA was extracted from the testis or muscle tissues of adult beetles or larvae preserved in absolute ethyl alcohol using the Wizard Genomic DNA Purification kit (Promega).

We amplified fragments of the COI gene (primers C1-J-2183 and L2-N-3014, Simon et al., 1994) and ITS region (primers 5.8S38F and ITS4col, Tanahashi & Hawes, 2016) to explore the phylogenetic relationships within and between the two species. COI was amplified by polymerase chain reaction (PCR) at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min, and a final extension for 7 min at 72°C. The ITS region was amplified using the same process, but with an annealing temperature of 50°C. The PCR products were purified using the Illustra ExoStar Clean-up kit (GE Healthcare).

Additionally, we used 65 COI and 5 ITS sequences for *P. delicatulus* and *P. kawadai*, and 9 COI and 2 ITS sequences for the outgroup (*P. akitaiorum* and *P. sugitai*) from previous studies (Kubota et al., 2010, 2011; Zhu et al., 2020). In total, we used 168 COI and 52 ITS sequences for analysis. The best-fit substitution model for COI and the ITS region were selected using jModelTest v.2.1.10 (Darriba et al., 2012) based on the Akaike information criterion (AIC).
Bayesian interference (BI) trees were constructed using MrBayes v.3.2.7 (Ronquist et al., 2012) for 100 million generations (sample frequency = 50,000) with Tracer v.1.7.1 (Rambaut et al., 2018). After discarding the first 10% of samples as burn-in, majority-rule consensus (MRC), trees were constructed by the sumt function in MrBayes. The final tree was visualized using FigTree v.1.4.2 (Rambaut, 2016). Maximum-likelihood (ML) trees were constructed using RAxML v.8.2.9 (Stamatakis, 2016) with the best-fit substitution model selected using 1000 bootstrap replications.

Divergence times were estimated using BEAST v.2.6.2 based on the strict molecular clock with a substitution rate of 1.77% per lineage in million years (Myr) for COI (Papadopoulou et al., 2010). The data consisted of only in-group taxa, and the topology was fixed to the ML tree. Markov Chain Monte Carlo analysis was performed using 10 million generations, sampling every 1000 generations. The convergence of the chains was confirmed using Tracer v.1.7.1. After discarding 10% of samples as burn-in, samples from the posterior distributions were summarized on a maximum clade credibility tree using TreeAnnotator v.1.10.5. FigTree v.1.4.2 was used to visualize the resulting tree.

Based on the molecular analysis results, we subdivided the populations of *P. kawadai* into two analysis units (see RESULTS). For *P. delicatulus*, we focused on one COI clade containing populations sympatric with *P. kawadai*, and subdivided this clade into two analysis units (i.e., sympatric or allopatric with *P. kawadai*).

### 2.2 Morphological analysis

For the morphological analysis, we assessed morphological external differentiation of *P. delicatulus* (central-to-northern Honshu) and

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**TABLE 1** Summary of environmental variables used in this study

| Code | Environmental variables                     | Unit |
|------|---------------------------------------------|------|
| Ele  | Elevation                                   | m    |
| Bio3 | Isothermality                                | –    |
| Bio4 | Temperature seasonality                      | –    |
| Bio8 | Mean temperature of the wettest quarter     | °C   |
| Bio12| Annual precipitation                         | mm   |
| Bio19| Precipitation of coldest quarter            | mm   |

**TABLE 2** Correlation for the environmental variables associated with Platycerus occurrence sites

| Code | Bio3 | Bio4 | Bio8 | Bio12 | Bio19 |
|------|------|------|------|-------|-------|
| Ele  | 0.75 | -0.45| -0.11| 0.21  | -0.53 |
| Bio3 | 1    |      |      |       |       |
| Bio4 | 1    |      |      |       |       |
| Bio8 | 1    |      |      |       |       |

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**FIGURE 4** Consensus tree based on majority rule (>50%) of Bayesian inference (BI) tree for *Platycerus delicatulus* and *Platycerus kawadai* in Japan based on ITS sequences. *Platycerus akitaorum* and *Platycerus sugitai* were used as the outgroup. Operational taxonomic units indicate the combination of “species” and “site number (number of individuals sharing the same haplotype)”. Numbers near the branches indicate nodal support (posterior probability in the BI tree (> 50%) and bootstrap probability in the maximum-likelihood (ML) tree (> 50%))
P. kawadai specimens collected from 2005 to 2020, which were deposited in the Forest Zoology Laboratory of the University of Tokyo. We focused on external body size and shape using traits related to ecological specialization. Those selected morphological traits in this study are often associated with adaptation processes as demonstrated by published literature (Hagge et al., 2021; Konuma et al., 2013; Okada & Miyatake, 2009). We firstly captured video images of specimens in dorsal view using a DP12 digital camera (Olympus, Tokyo) attached to an SZ10 stereoscopic microscope (Olympus). Then, we measured the eight morphological traits illustrated in Figure 2 from each habitus image using Photoshop software (Adobe, San Jose, CA) on a personal computer. We measured the trait lengths of adult beetles, including 213 specimens (111 males and 102 females) of P. delicatulus (23 sites for male and 24 sites for female) and 253 specimens (142 males and 113 females) of P. kawadai (26 sites for male and 22 sites for female).

To obtain a general view of the morphological differences among the populations, we first log-transformed all trait measurements and...
performed a principal components analysis (PCA) using the procomp function in R v.3.6.3 (R Core Team, 2013) and visualized the results using "ggplot2" (Wickham & Wickham, 2007). To examine whether the two species differed in their morphological traits, we compared the principal component (PC) 1 and PC2 between two populations for each sex of each species. Then, we tested for body length (BL) differences between and within species and between the sexes using analysis of variance (ANOVA) and Tukey’s HSD post hoc tests; BL was used as the measure for analysis, as it provides greater reproducibility than an axis derived from PCA (Barton et al., 2011).

For genital morphology, although we observed little difference in endophallic structure between P. delicatulus and P. kawadai (Figure 3), which may be concerning for reproductive isolation, we found no difference among populations within each species. Quantitatively assessing the membranous part of the endophallus is difficult, so we did not consider genital morphological variation.

2.3 | Environmental analysis

Environmental data were downloaded from the Worldclim database (v.1.4; http://www.worldclim.org; Hijmans et al., 2005) at a resolution of 30 arc seconds. A total of 99 occurrences of nonduplicated records (55 for P. delicatulus and 44 for P. kawadai) were obtained...
from field surveys and previous research (Zhang & Kubota, 2021). Next, we extracted 19 bioclimatic variables for each sampling location and tested multicollinearity among these variables. We excluded bioclimatic variables with a Pearson's correlation coefficient \(|r| > .8\). Accordingly, we retained six climatic variables for subsequent analysis: Elevation (Ele), isothermality (Bio3), temperature seasonality (Bio4), mean temperature of wettest quarter (Bio8), annual precipitation (Bio12), and precipitation of coldest quarter (Bio19) (Tables 1 and 2).

To quantify the environmental niches of *P. delicatulus* and *P. kawadai* populations, we used two statistical approaches. First, PCA was performed on the environmental variables using procomp function in R v.3.6.3 (R Core Team, 2013) and visualized using “ggplot2” (Wickham & Wickham, 2007). Second, we compared the environmental niche spaces of the species using \(n\)-dimensional hypervolumes analyses (Hutchinson, 1957), which were conducted using the “hypervolume” R package (Blonder et al., 2018). We constructed the hypervolumes using the six retained variables for the major populations. All environmental variables were natural log-transformed for analysis. All hypervolumes were created using the Gaussian kernel density estimator method with the default Silverman bandwidth estimator (Blonder et al., 2014, 2018). To compare hypervolumes among environmental variables, we quantified the pairwise overlap between populations, using the Jaccard and Sorensen similarity indexes following Blonder et al. (2018).

### 2.4 Correlations between morphology and environmental niche

We conducted Mantel tests and partial Mantel tests using the “vegan” R package to test correlation between the morphological and environmental distances of *P. delicatulus* and *P. kawadai* (Oksanen et al., 2013). Morphological distance was calculated as the Euclidean pairwise distance of BL between localities because BL is considered as an important trait for resource competition and reproductive interference (Okuzaki, 2021; Takami & Sota, 2007). Geographic distance was assessed as the Euclidean distance of latitude and longitude between localities. For environmental distance, we firstly scaled the six environmental variables prior to creating a distance matrix using

![FIGURE 7](image-url) Principal component analysis plots of morphological data showing differentiation between populations of *Platycerus delicatulus* and *Platycerus kawadai*. Ellipses represent the 95% confidence intervals.
FIGURE 8  Morphological differentiation between populations along the first two principal components (PC1, a−d; PC2, e−h) for Platycerus delicatulus male (a, e) and female (b, f) individuals, and P. kawadai male (c, g) and female (d, h) individuals. Student’s t-test results are also shown. *, p < .05; **, p < .01; ***, p < .001

TABLE 3  Principal component analysis (PCA) loading scores for morphological traits used to evaluate the morphological differentiation for males of Platycerus delicatulus

| Morphological traits | Male | Female |
|---------------------|------|--------|
|                     | PC1  | PC2    | PC1  | PC2    |
| Head width (HW)     | 0.93 | -0.10  | 0.83 | -0.05  |
| Pronotum width (PW) | 0.94 | -0.16  | 0.94 | -0.11  |
| Elytra width (EW)   | 0.75 | -0.22  | 0.81 | -0.15  |
| Head length (HL)    | 0.63 | 0.68   | 0.41 | 0.72   |
| Pronotum length (PL)| 0.91 | -0.15  | 0.86 | 0.01   |
| Elytra length (EL)  | 0.65 | 0.36   | 0.92 | -0.17  |
| Body length (BL)    | 0.95 | -0.09  | 0.96 | -0.06  |
| Mandible length (ML)| 0.86 | -0.03  | 0.21 | 0.83   |
| Eigenvalue           | 5.59 | 0.71   | 4.97 | 1.28   |
| % of variance        | 69.92| 8.86   | 62.07| 16.02  |

Note: The trait that contributed the most is highlighted in bold on PC1.

TABLE 4  Principal component analysis (PCA) loading scores for morphological traits used to evaluate the morphological differentiation of Platycerus kawadai

| Morphological traits | Male | Female |
|---------------------|------|--------|
|                     | PC1  | PC2    | PC1  | PC2    |
| Head width (HW)     | 0.88 | 0.32   | 0.68 | 0.31   |
| Pronotum width (PW) | 0.92 | -0.10  | 0.91 | -0.15  |
| Elytra width (EW)   | 0.68 | -0.57  | 0.78 | -0.33  |
| Head length (HL)    | 0.80 | 0.18   | 0.48 | 0.68   |
| Pronotum length (PL)| 0.86 | -0.13  | 0.78 | -0.12  |
| Elytra length (EL)  | 0.89 | -0.19  | 0.88 | -0.21  |
| Body length (BL)    | 0.97 | -0.07  | 0.96 | -0.03  |
| Mandible length (ML)| 0.68 | 0.60   | 0.18 | 0.85   |
| Eigenvalue           | 5.66 | 0.87   | 4.47 | 1.48   |
| % of variance        | 70.79| 10.96  | 55.94| 18.49  |

Note: The trait that contributed the most is highlighted in bold on PC1.
scale function, because the environmental variables were all measured using different metrics that are not comparable to each other. Then, we calculated Euclidean pairwise distance of the environmental variables between sites using dist function (Oksanen et al., 2019). Finally, the significances between the geographic distance and morphological distance or between environmental and morphological distance were assessed by running 10,000 permutations. The partial Mantel test was used to determine whether morphological distance was correlated with environmental distance while controlling for the effect of geographic distance (Morpho, Env | Geo) based on Pearson correlation coefficients. Regression analysis was used to describe the relationship of the residual morphological values vs. residual geographic values and residual morphological values vs. residual environmental values for populations of each species.

3 | RESULTS

3.1 | Phylogenetic relationship between species

We sequenced 784 bp of the COI gene and 730–732 bp of the ITS region. These sequences were deposited in GenBank (DDBJ accession numbers: LC651809–LC651901 for the COI gene, and LC651902–LC651946 for the ITS region). The best-fit models were GTR + G for COI and GTR + G for the ITS region.

Based on the ITS region, *P. delicatulus* and *P. kawadai* constitute an independent distant monophyletic group, which aligned with the morphologically identified species units. *P. delicatulus* was subdivided into a Honshu and Shikoku population and a Kyushu population (Figure 4).

Two major clades were obtained based on the COI gene (Figure 5). Clade I was composed of entirely of *P. kawadai*, whereas Clade II contained both species. Clade II-a-1 composed of *P. kawadai* based on morphology and was assumed to contain the offspring of a population that receive mitochondrial genes from *P. delicatulus* via the introgressive hybridization. Clades II-a-2, II-a-3, and II-b were composed mainly of *P. delicatulus*. However, a male *P. kawadai* collected at Site 97 was in Clade II-a-2, whereas another individual from that site belonged to Clade I (Figure 5).

The divergence times of *P. delicatulus* and *P. kawadai* populations were estimated based on the COI gene (Figure 6). The estimated divergence time between Clades I and II (representing the speciation between *P. delicatulus* and *P. kawadai*) was 1.16 Mya. Clade II was subdivided into Clade II-a (generally, *P. delicatulus*: Honshu, Shikoku, and northern Kyushu) and Clade II-b (*P. delicatulus*: southern Kyushu) at 0.96 Mya. The introgressive hybridization that was the origin of Clade II-a-1 occurred approximately 0.74 Mya. In the recent past, an introgressive hybridization occurred at Site 97 (Figures 5 and 6).

For subsequent analyses, in the context of the interspecific relationship and intraspecific divergence, we subdivided *P. kawadai* populations into two analysis units: “No introgression” population (Clade I) and “Introgression” population (Clade II-a-1) based on the molecular results. In this classification, we excluded the population at Site 97 with a *P. kawadai* sample exhibiting the introgression type for COI gene from *P. delicatulus*. It is a very rare case because all other samples from the same mountain range (Akaishi Mountains) including Site 97 exhibited no introgression type. Sites at which no genetic samples were collected were assigned to the category of the closest site at which genetic samples were collected. We subdivided *P. delicatulus* populations belonging to Clade II-a-2 into “Sympatric” population and “Allopatric” population. Sympatric population range covers whole range of *P. kawadai*, whereas both species cannot be always collected at the same site (Figure 1, Appendix 1). In the following part, we examined the morphological differentiation among these analysis units of two species.
3.2 | Morphological differentiation

Examinations of morphological variation in eight traits by PCA indicated differentiation between Allopatric and Sympatric populations of *P. delicatulus*, as well as between No introgression and Introggression populations of *P. kawadai* mainly along the PC1 axis (Figure 7). Specifically, male and female populations of *P. delicatulus* were mainly discriminated by the first principal component (PC1), which explained 69.92% and 62.07% of the variance, respectively. For *P. kawadai*, PC1 explained 70.79% and 55.94% of the total variance for male and female, respectively. The significant difference between the populations in PC2 was detected only for *P. delicatulus* males (Figure 8). In this case, the eigenvalue of PC2 was 0.71 and the highest loading score for PC2 was 0.68 of head length (HL) (Table 3). PC2 and HL could not sufficiently explain the morphological differentiation between the populations. On the other hand, the significant difference between populations in PC1 was detected for most studied species and sexes except for *P. kawadai* males (Figure 8). BL exhibited the highest loading scores on the first axis PC1 (0.95–0.97) in both species and sexes (Tables 3 and 4). Additionally, BL showed a significant level of differentiation between Allopatric and Sympatric populations of *P. delicatulus*, as well as between No introgression and Introggression populations of *P. kawadai* for both male and female individuals (*p* < .001, ANOVA; Figure 9), but we found no significant differentiation in BL between Allopatric populations of *P. delicatulus* and Introggression populations of *P. kawadai* for males (Figure 9a).
On the other hand, female BL varied significantly between the two species (Figure 9b). Sympatric population of *P. delicatulus* and No introgression population of *P. kawadai* showed the highest and lowest value, respectively (Figure 9).

### 3.3 Environmental niche

For *P. delicatulus*, we found the PCA results suggested that Sympatric population had a narrower environmental space than that of Allopatric population, especially in terms of elevation, temperature seasonality (Bio4), and mean temperature in wettest quarter (Bio8) (Figure 10a; Table 5). Two principal components (PC) explained 44.6% (PC1) and 29.28% (PC2) of the variation between populations of *P. delicatulus*. For *P. kawadai*, two primary principal components (PC) accounted for 47.2% (PC1) and 26.8% (PC2) of the total variance (Figure 10b). No introgression population exhibited higher temperature seasonality and lower mean temperature of wettest quarter, favoring less precipitation (Bio12 and Bio19) and a wider elevation compared with the Introggression population of *P. kawadai* (Table 5).

The multidimensional variations in the environmental space of both species are shown as niche hypervolumes in Figures 11 and 12, illustrating that the populations occupied different ecological spaces with relatively little overlap. For *P. delicatulus*, the niche hypervolume was much greater for the Allopatric population than for the Sympatric population, and they overlapped slightly (Sørensen similarity = 0.057, Jaccard similarity = 0.029; Figure 11). For *P. kawadai*, the Sørensen and Jaccard similarity index values of the hypervolumes were 0.135 and 0.072 in No introgression and Introgression populations, respectively. Generally, Bio4 did not overlapped between the populations (Figure 12).

### 3.4 Correlation between morphological and environmental niche

For *P. delicatulus*, simple Mantel tests showed that the morphological distance between populations was not significantly correlated with environmental (male, $p = .104$; female, $p = .283$) or geographic distances (male, $p = .119$; female, $p = .315$) (Table 6). Morphological distance was not related with environmental distance after controlling for the effect of geographic distance (male, $p = .102$; female, $p = .588$, Figure 13b,d)
**FIGURE 12** Hypervolumes obtained from multidimensional kernel density estimation of the studied population (Allopatric and Sympatric population) of *Platycerus kawadai* based on weakly correlated environmental variables. The larger colored dots represent species centroids.

**TABLE 6** Single and partial Mantel test results based on morphological, environmental, and geographic distances between occurrence sites of *Platycerus delicatulus* and *P. kawadai*

| Comparison                        | Sex      | *P. delicatulus* | *P. kawadai* |
|-----------------------------------|----------|------------------|--------------|
|                                   |          | *r*  | *p*-Value | *r*  | *p*-Value |
| Single Mantel tests               |          |     |          |     |          |
| Morphological and environmental   | Males    | .160 | .104      | .170 | .006      |
|                                   | Females  | .048 | .283      | .331 | .007      |
| Morphological and geographic      | Males    | .062 | .119      | .469 | <.001     |
|                                   | Females  | .024 | .315      | .249 | .003      |
| Partial Mantel tests              |          |     |          |     |          |
| Morphological and environmental | Males    | .170 | .102      | .009 | .470      |
|                                  | Females  | .058 | .241      | .059 | .698      |
| Morphological and geographic     | Males    | .059 | .608      | .443 | <.001     |
|                                  | Females  | .032 | .588      | .316 | .010      |

Note: Bold values denote statistical significance at the *p* < .05 level.
based on the partial Mantel test results. On the other hand, for *P. kawadai*, morphological distance was significantly correlated with the environmental (male, \( p = .005 \); female, \( p = .03 \)) and geographic distances (male, \( p < .001 \); female, \( p = .003 \)) (Table 6). Morphological distances were not significantly correlated with environmental distances after controlling for geographic distances using partial Mantel tests for *P. kawadai* (male, \( p = .470 \); female, \( p = .698 \), Figure 14a,c), however, morphological distance was significantly correlated with geographic distances after controlling for environmental distance in the same manner (male, \( p < .001 \); female, \( p = .010 \), Figure 14b,d).

4 | DISCUSSION

4.1 | Phylogeographic history of the two related species

The genetic sample collection sites of the two species cover almost their entire distribution ranges (Appendix 2). Phylogenetic analyses based on the ITS region suggested that *P. delicatulus* and *P. kawadai* are each essentially monophyletic (Figure 4). This result aligns with the phylogenetic results of their yeast symbionts (Kubota et al., 2020). Since the ancestral branches of *P. delicatulus* diverged in western Japan, it is likely that the two species were separated and speciated in western (*P. delicatulus*) and central (*P. kawadai*) Japan approximately 1.16 Mya (Figures 5 and 6). Following that speciation event, *P. delicatulus* was separated into two clades (Clade II-a: Honshu, Shikoku, and northern Kyushu; and Clade II-b: southern Kyushu in COI) approximately 0.96 Mya. The Clade II-a population of *P. delicatulus* expanded eastward, and hybridized with *P. kawadai* after 0.74 Mya, which resulted in portion of *P. kawadai* forming a clade (Clade II-a-1: Introggression population) nested within the *P. delicatulus* clade (Clade II). Since then, introgressive hybridization appears to have occurred very rarely between the two species (Figures 5 and 6). Moreover, in terms of the direction of introgression, morphological similarity may have resulted in a relatively higher probability of introgression from *P. delicatulus* to *P. kawadai* than in the reverse direction. *P. delicatulus* females and *P. kawadai* males may occasionally mate with each other because females of *P. delicatulus* have a larger body size than *P. kawadai* and mitochondrial genes are maternally inherited only. Based on our observation, males of *Platycerus* species always try
to mate immediately with any female during the reproductive season. When there is a chance of heterospecific mating, interspecific differences in body size and genitalia size may work as pre-mating and mechanical isolation mechanisms, respectively (Kubota & Sota, 1998; Takami & Sota, 2007; Okuzaki, 2021). A similar phylogeographic pattern has been documented in other beetles (Kosuda et al., 2016; Takami et al., 2007; Zhang & Sota, 2007).

These results indicated that No introgression population and Introgression population of P. kawadai differed mainly in terms of COI, but they cannot be distinguished using ITS sequences. Possible explanations for the mitochondrial–nuclear discordance could be associated with sex-biased dispersal, mating, and offspring production (Bonnet et al., 2017). Genetic drift is ubiquitous in populations and can interact with many of the above processes to increase discordance between mitochondrial and nuclear genes (Toews & Brelsford, 2012). But it is difficult to explain the essential topological difference between the COI and ITS phylogenies just for these reasons. Another possible evolutionary scenario for such a discordance is the incomplete lineage sorting following the ancestral polymorphism of mitochondrial gene (Funk & Omland, 2003). However, it is unlikely that the ancestor of P. kawadai had possessed both mitochondrial Clades I and II-a-1 because Clade II-a-1 had occurred in a P. delicatulus type subclade (Clade II-a) after initial geographical differentiation within P. delicatulus. An alternative and more likely scenario is historical mitochondrial introgression following the range expansion of these species. Because Clade II-a-1 was diverged from a P. delicatulus type clade around 0.74 Mya, the replacement by an introgressive clade seems to be very rare and only one replacement is recognized.

4.2 | Factors affecting morphological differences among Platycerus populations within species

In this study, we constructed intraspecific analysis units of two Platycerus species based on interspecific ranges and evolutionary dynamics, and then evaluated the factors affecting the morphological differences within each species. Among the eight morphological traits shown in Figure 3, BL was the most effective variable for explaining morphological variation (Figure 7). Meanwhile, the results of the n-dimensional hypervolume analysis revealed environmental heterogeneity among populations. We tested whether the morphological
variation across populations was better explained by geographic dis-
tance with dispersal or by environmental filtering for studied species.

For *P. delicatulus*, the morphological (BL) distance among col-
lection sites was not correlated with environmental factors or with
geographic distance, and therefore these factors could not explain
the morphological divergence between Allopatric and Sympatric
populations. The latter population is larger than the former, and
likely arose via character displacement against *P. kawada* (Figure 9).
As *P. delicatulus* and *P. kawada* are capable of mating, the putative
character displacement may be caused by reproductive interference
other than the resource competition. Overall, our results suggest
that interspecific interaction has played a major role in driving the
morphological differentiation of *P. delicatulus* populations.

For *P. kawada*, morphological distance was correlated with
geographic distance after controlling for environmental distance
(Table 6). This result suggests that geographic distance (i.e., low
dispersal ability) might have led to morphological differentiation.
Therefore, dispersal is assumed to drive the morphological diversi-
fication of populations. Meanwhile, dispersal ability could influence
range limits and gene flow among populations, which may be associ-
ated with niche differentiation. In addition, previous studies showed
that morphological adaptation to local ecology can also have resulted
from phenotypic plasticity or from genetic differences among pop-
ulations (Borokini et al., 2021; Ghalambor et al., 2007; Kunz et al.,
2022; Price et al., 2003; Schmid & Guillaume, 2017). Although phen-
otypic plasticity has been documented in response to variations in
multiple environmental variables (Chevin & Lande, 2015; Gratani,
2014; Lande, 2009; Wang et al., 2021), we found morphological
distance was not correlated with environmental distance after con-
trolling for geographic distance (Table 6). Thus, environmental fac-
tors are unlikely to be responsible for the observed morphological
differentiation in *P. kawada*. However, we cannot exclude the possi-
bility that genetic divergence, such as that achieved via genetic drift
and intra- and interspecific gene flow, promoted the morphological
divergence. Further studies are required to verify whether this possi-
bility would explain the morphological differentiation among pop-
ulations of *P. kawada*.

Populations often experience different environmental condi-
tions, leading to the evolution of different phenotypes to maximize
fitness (Freudiger et al., 2021; Jones et al., 2021). Most studies have
shown that body size is affected by environmental filtering and food
availability, which exhibit trade-off relationships (Dmitriev, 2011;
Konuma et al., 2011; Runemark et al., 2015). Our results showed that
intraspecific morphological variations in *P. delicatulus* and *P. kawada*
were related to interspecific interaction and geographic distance, re-
spectively. These results indicated divergence between populations
in directions of morphological variation and provided significant ins-
sights into species adaptation processes.

In conclusion, we integrated morphological, environmental, and
molecular data across the geographic ranges of two species to in-
vestigate the ecological–evolutionary processes that may drive
divergence processes among populations and across geography.
We found that morphological and ecological niche differentiation
within species may be driven by interspecific interaction, as well as
dispersal ability. These differentiations may associate with special-
ization for habitat preference. Our results elucidate ecological pro-
cess across species’ distributions through adaptation and plasticity
in natural systems. Evidence of divergence between populations
provides a useful reference for conservation strategies to enhance
potential for adaptive response to the challenging climate changes.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Sheng-Nan Zhang: Conceptualization (lead); Data curation (equal);
Formal analysis (lead); Investigation (equal); Methodology (lead);
Writing – original draft (lead); Writing – review & editing (support-
ing). Köhe Kubota: Conceptualization (supporting); Data curation
(equal); Formal analysis (supporting); Funding acquisition (lead);
Investigation (equal); Methodology (supporting); Project administra-
tion (lead); Resources (lead); Supervision (lead); Writing – original
draft (supporting); Writing – review & editing (lead).

DATA AVAILABILITY STATEMENT

Sequence data are available at the DDBJ database under access-
cession numbers LC651809–LC651901 for the COI gene, and
LC651902–LC651946 for the ITS region, https://www.ddbj.nig.
ac.jp/ddbj/index-e.html.

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APPENDIX 1

Occurrence records of Platycerus examined

| Species        | Analysis unit | Elevation (m) | Latitude (°) | Longitude (°) | Site No. |
|----------------|---------------|---------------|--------------|---------------|----------|
| *P. delicatulus* | Allopatric    | 370           | 41.15        | 140.38        | 1        |
| *P. delicatulus* | Allopatric    | 700           | 40.50        | 140.83        | 2        |
| *P. delicatulus* | Allopatric    | 410           | 40.49        | 140.93        | 3        |
| *P. delicatulus* | Allopatric    | 430           | 40.51        | 140.97        | 4        |
| *P. delicatulus* | Allopatric    | 1000          | 38.52        | 139.73        | 5        |
| *P. delicatulus* | Allopatric    | 700           | 38.28        | 140.46        | 6        |
| *P. delicatulus* | Allopatric    | 640           | 38.48        | 140.01        | 7        |
| *P. delicatulus* | Allopatric    | 740           | 38.53        | 139.96        | 8        |
| *P. delicatulus* | Allopatric    | 460           | 38.21        | 139.85        | 9        |
| *P. delicatulus* | Allopatric    | 880           | 38.14        | 140.51        | 10       |
| *P. delicatulus* | Allopatric    | 1000          | 37.06        | 139.48        | 11       |
| *P. delicatulus* | Allopatric    | 800           | 37.09        | 139.59        | 12       |
| *P. delicatulus* | Allopatric    | 960           | 36.93        | 140.28        | 13       |
| *P. delicatulus* | Allopatric    | 1300          | 36.87        | 139.40        | 14       |
| *P. delicatulus* | Allopatric    | 1280          | 36.75        | 139.44        | 15       |
| *P. delicatulus* | Allopatric    | 900           | 36.75        | 138.83        | 16       |
| *P. delicatulus* | Allopatric    | 1220          | 36.67        | 138.67        | 17       |
| *P. delicatulus* | Allopatric    | 1130          | 36.48        | 138.88        | 18       |
| *P. delicatulus* | Allopatric    | 1300          | 36.77        | 138.82        | 19       |
| *P. delicatulus* | Allopatric    | 1100          | 36.85        | 137.83        | 20       |
| *P. delicatulus* | Allopatric    | 1250          | 36.38        | 137.75        | 21       |
| *P. delicatulus* | Allopatric    | 1320          | 36.14        | 136.73        | 22       |
| *P. delicatulus* | Allopatric    | 1030          | 35.52        | 136.41        | 23       |
| *P. delicatulus* | Allopatric    | 550           | 34.46        | 136.24        | 24       |
| *P. delicatulus* | Allopatric    | 1410          | 34.38        | 136.09        | 25       |
| *P. delicatulus* | Allopatric    | 1300          | 34.35        | 136.21        | 26       |
| *P. delicatulus* | Allopatric    | 1200          | 34.32        | 136.20        | 27       |
| *P. delicatulus* | Allopatric    | 1200          | 34.21        | 136.12        | 28       |
| *P. delicatulus* | Allopatric    | 1520          | 34.19        | 136.10        | 29       |
| *P. delicatulus* | Allopatric    | 1150          | 34.22        | 135.98        | 30       |
| *P. delicatulus* | Allopatric    | 1250          | 33.90        | 135.65        | 31       |
| *P. delicatulus* | Allopatric    | 1250          | 34.15        | 135.65        | 32       |
| *P. delicatulus* | Allopatric    | 690           | 35.35        | 135.74        | 33       |
| *P. delicatulus* | Sympatric     | 1260          | 36.41        | 138.67        | 34       |
| *P. delicatulus* | Sympatric     | 1400          | 36.20        | 138.64        | 35       |
| *P. delicatulus* | Sympatric     | 1300          | 35.94        | 138.80        | 36       |
| *P. delicatulus* | Sympatric     | 1100          | 35.91        | 138.82        | 37       |
| *P. delicatulus* | Sympatric     | 780           | 35.92        | 138.84        | 38       |
| *P. delicatulus* | Sympatric     | 1420          | 35.85        | 138.98        | 39       |
| *P. delicatulus* | Sympatric     | 1450          | 35.74        | 139.02        | 40       |
| *P. delicatulus* | Sympatric     | 1210          | 35.48        | 139.17        | 41       |
| *P. delicatulus* | Sympatric     | 1567          | 35.47        | 139.16        | 42       |
| *P. delicatulus* | Sympatric     | 1587          | 35.51        | 139.07        | 43       |
| *P. delicatulus* | Sympatric     | 1400          | 35.51        | 139.05        | 44       |
| Species          | Analysis unit | Elevation (m) | Latitude (°) | Longitude (°) | Site No. |
|------------------|---------------|---------------|--------------|---------------|----------|
| P. delicatulus   | Sympatric     | 1570          | 35.69        | 138.88        | 45       |
| P. delicatulus   | Sympatric     | 1100          | 35.78        | 138.77        | 46       |
| P. delicatulus   | Sympatric     | 1200          | 35.86        | 138.56        | 47       |
| P. delicatulus   | Sympatric     | 1550          | 35.38        | 138.53        | 48       |
| P. delicatulus   | Sympatric     | 1420          | 35.32        | 138.36        | 49       |
| P. delicatulus   | Sympatric     | 1480          | 36.90        | 138.49        | 50       |
| P. delicatulus   | Sympatric     | 1240          | 36.41        | 138.60        | 51       |
| P. delicatulus   | Sympatric     | 1550          | 35.39        | 137.99        | 52       |
| P. delicatulus   | Sympatric     | 1620          | 35.13        | 138.04        | 53       |
| P. delicatulus   | Sympatric     | 1180          | 35.23        | 137.99        | 54       |
| P. delicatulus   | Sympatric     | 1260          | 35.12        | 137.90        | 55       |
| P. delicatulus   | (Others)      | 1050          | 35.25        | 134.39        | 56       |
| P. delicatulus   | (Others)      | 1100          | 35.19        | 133.82        | 57       |
| P. delicatulus   | (Others)      | 970           | 35.35        | 133.54        | 58       |
| P. delicatulus   | (Others)      | 1100          | 34.69        | 132.19        | 59       |
| P. delicatulus   | (Others)      | 1080          | 34.50        | 132.13        | 60       |
| P. delicatulus   | (Others)      | 1220          | 33.92        | 134.34        | 61       |
| P. delicatulus   | (Others)      | 1120          | 33.91        | 134.29        | 62       |
| P. delicatulus   | (Others)      | 1030          | 33.92        | 134.29        | 63       |
| P. delicatulus   | (Others)      | 1220          | 33.88        | 134.11        | 64       |
| P. delicatulus   | (Others)      | 1320          | 33.87        | 134.09        | 65       |
| P. delicatulus   | (Others)      | 1140          | 33.94        | 132.94        | 66       |
| P. delicatulus   | (Others)      | 1430          | 33.75        | 133.15        | 67       |
| P. delicatulus   | (Others)      | 1480          | 33.48        | 133.02        | 68       |
| P. delicatulus   | (Others)      | 1150          | 33.19        | 132.61        | 69       |
| P. delicatulus   | (Others)      | 960           | 33.48        | 130.93        | 70       |
| P. delicatulus   | (Others)      | 740           | 33.46        | 130.91        | 71       |
| P. delicatulus   | (Others)      | 1100          | 33.28        | 131.40        | 72       |
| P. delicatulus   | (Others)      | 880           | 33.12        | 131.29        | 73       |
| P. delicatulus   | (Others)      | 1620          | 32.58        | 131.11        | 74       |
| P. delicatulus   | (Others)      | 1250          | 32.16        | 130.93        | 75       |
| P. delicatulus   | (Others)      | 1400          | 32.30        | 131.43        | 76       |
| P. delicatulus   | (Others)      | 1320          | 32.28        | 131.43        | 77       |
| P. delicatulus   | (Others)      | 1250          | 31.94        | 130.85        | 78       |
| P. delicatulus   | (Others)      | 700           | 33.00        | 130.07        | 79       |
| P. delicatulus   | (Others)      | 900           | 32.98        | 130.09        | 80       |
| P. delicatulus   | (Others)      | 970           | 32.96        | 130.08        | 81       |
| P. delicatulus   | (Others)      | 1200          | 32.76        | 130.29        | 82       |
| P. kawadai       | No introgression | 1400       | 36.44        | 138.64        | 83       |
| P. kawadai       | No introgression | 1260       | 36.41        | 138.67        | 84       |
| P. kawadai       | No introgression | 1400       | 36.20        | 138.64        | 85       |
| P. kawadai       | No introgression | 1300       | 35.94        | 138.80        | 86       |
| P. kawadai       | No introgression | 1120       | 35.91        | 138.82        | 87       |
| P. kawadai       | No introgression | 1490       | 35.90        | 138.95        | 88       |
| P. kawadai       | No introgression | 1400       | 35.87        | 139.09        | 89       |
| Species       | Analysis unit     | Elevation (m) | Latitude (°) | Longitude (°) | Site No. |
|---------------|-------------------|---------------|--------------|---------------|----------|
| P. kawadai    | No introgression  | 1400          | 35.71        | 138.83        | 86       |
| P. kawadai    | No introgression  | 1550          | 35.56        | 138.75        | 87       |
| P. kawadai    | No introgression  | 1569          | 35.42        | 138.69        | 88       |
| P. kawadai    | No introgression  | 1550          | 35.38        | 138.53        | 48       |
| P. kawadai    | No introgression  | 1480          | 35.32        | 138.35        | 49       |
| P. kawadai    | No introgression  | 1400          | 35.64        | 138.35        | 89       |
| P. kawadai    | No introgression  | 1330          | 36.91        | 138.48        | 50       |
| P. kawadai    | No introgression  | 1350          | 36.11        | 138.65        | 90       |
| P. kawadai    | No introgression  | 1300          | 36.31        | 138.08        | 91       |
| P. kawadai    | No introgression  | 1550          | 35.39        | 137.99        | 52       |
| P. kawadai    | No introgression  | 1500          | 35.57        | 138.12        | 92       |
| P. kawadai    | No introgression  | 1600          | 35.57        | 138.08        | 93       |
| P. kawadai    | No introgression  | 1640          | 35.55        | 138.09        | 94       |
| P. kawadai    | No introgression  | 1600          | 35.44        | 137.96        | 95       |
| P. kawadai    | No introgression  | 1600          | 35.20        | 137.98        | 96       |
| P. kawadai    | No introgression  | 1600          | 35.24        | 137.96        | 97       |
| P. kawadai    | No introgression  | 1260          | 35.12        | 137.90        | 98       |
| P. kawadai    | Introgression     | 1460          | 35.52        | 138.97        | 99       |
| P. kawadai    | Introgression     | 1240          | 35.44        | 139.23        | 100      |
| P. kawadai    | Introgression     | 1210          | 35.48        | 139.17        | 41       |
| P. kawadai    | Introgression     | 1567          | 35.47        | 139.16        | 42       |
| P. kawadai    | Introgression     | 1600          | 35.48        | 139.10        | 101      |
| P. kawadai    | Introgression     | 1587          | 35.51        | 139.07        | 43       |
| P. kawadai    | Introgression     | 1673          | 35.49        | 139.14        | 102      |
| P. kawadai    | Introgression     | 1292          | 35.48        | 139.03        | 103      |
| P. kawadai    | Introgression     | 1400          | 35.51        | 139.05        | 44       |
| P. kawadai    | Introgression     | 1379          | 35.46        | 138.98        | 104      |
| P. kawadai    | Introgression     | 1320          | 35.40        | 138.92        | 105      |
| P. kawadai    | Introgression     | 1350          | 35.39        | 138.89        | 106      |
| P. kawadai    | Introgression     | 1420          | 35.23        | 139.02        | 107      |
| P. kawadai    | Introgression     | 1350          | 35.23        | 139.02        | 108      |
| P. kawadai    | Introgression     | 1299          | 34.86        | 139.02        | 109      |
| P. kawadai    | Introgression     | 1406          | 34.86        | 139.00        | 110      |
| P. kawadai    | Introgression     | 1200          | 34.85        | 138.96        | 111      |
| P. kawadai    | Introgression     | 1150          | 34.84        | 138.96        | 112      |
| P. kawadai    | Introgression     | 1013          | 34.84        | 138.89        | 113      |
| P. kawadai    | Introgression     | 1000          | 34.88        | 138.88        | 114      |
| P. akitaorum  | Introgression     | 1420          | 34.36        | 136.09        | 115      |
| P. akitaorum  | Introgression     | 1520          | 34.19        | 136.10        | 29       |
| P. akitaorum  | Introgression     | 1450          | 34.27        | 135.94        | 116      |
| P. akitaorum  | Introgression     | 1820          | 34.18        | 135.91        | 117      |
| P. sugitai    |                | 1220          | 33.92        | 134.34        | 118      |
| P. sugitai    |                | 1120          | 33.91        | 134.29        | 119      |
| P. sugitai    |                | 1320          | 33.87        | 134.09        | 65       |
| P. sugitai    |                | 1560          | 33.87        | 133.37        | 120      |
| P. sugitai    |                | 1520          | 33.76        | 133.14        | 121      |
## APPENDIX 2

### Samples of Platycerus used for morphological and genetic analyses

| Species       | Analysis Unit | Site No. | Number examined | Morphology | Genetic region | Accession No. of DDBJ |
|---------------|---------------|---------|----------------|------------|----------------|-----------------------|
| *P. delicatulus* | Allopatric    | 2       | 3 1            | 3 1        | COI ITS       | AB609374 LC651902    |
| *P. delicatulus* | Allopatric    | 3       | 1 1            | 2 1        | LC651811 LC651903 |
| *P. delicatulus* | Allopatric    | 9       | 3              | AB609375   |
| *P. delicatulus* | Allopatric    | 10      | 3 3            | AB426942   |
| *P. delicatulus* | Allopatric    | 11      | 1              | AB426943   |
| *P. delicatulus* | Allopatric    | 12      | 1 1            | AB426944   |
| *P. delicatulus* | Allopatric    | 13      | 16 8           | AB609378 LC651904 |
| *P. delicatulus* | Allopatric    | 14      | 2 2            | LC651813   |
| *P. delicatulus* | Allopatric    | 15      | 5              | AB609380   |
| *P. delicatulus* | Allopatric    | 16      | 6 4            | LC651814 LC651905 |
| *P. delicatulus* | Allopatric    | 18      | 1 3            | LC651817 LC651906 |
| *P. delicatulus* | Allopatric    | 19      | 1 1            | LC651818 LC651907 |
| *P. delicatulus* | Allopatric    | 21      | 2 2            | LC651820 LC651907 |
| *P. delicatulus* | Allopatric    | 22      | 1              | AB609381   |
| *P. delicatulus* | Allopatric    | 23      | 1              | AB426951   |
| *P. delicatulus* | Allopatric    | 24      | 9 8            | AB426952   |
| *P. delicatulus* | Allopatric    | 29      | 1 2            | AB609382 LC651908 |
| *P. delicatulus* | Allopatric    | 31      | 1              | LC651823   |
| *P. delicatulus* | Allopatric    | 33      | 1 1            | AB609383   |
| *P. delicatulus* | Allopatric    | 34      | 5 7            | AB426945   |
| *P. delicatulus* | Allopatric    | 35      | 1              | AB426946   |
| *P. delicatulus* | Allopatric    | 36      | 20 20          | AB426947   |

(Continues)
| Species         | Analysis Unit | Site No. | Number examined | Morphology | Genetic region | Accession No. of DDBJ |
|-----------------|---------------|----------|----------------|------------|----------------|----------------------|
| *P. delicatulus* | Sympatric     | 39       | 5              | 5          | 3              | LC651828             |
|                 |               |          |                |            |                | LC651829             |
|                 |               |          |                |            |                | LC651830             |
| *P. delicatulus* | Sympatric     | 40       | 3              | 4          | 3              | LC651831             |
|                 |               |          |                |            |                | LC651832             |
|                 |               |          |                |            |                | LC651833             |
| *P. delicatulus* | Sympatric     | 44       | 20             | 9          | 1              | 2                    | LC651834             | LC651911             |
|                 |               |          |                |            |                |                      | LC651912             |
| *P. delicatulus* | Sympatric     | 46       |                | 1          |                |                      |
| *P. delicatulus* | Sympatric     | 47       | 4              | 8          | 3              | AB426948             |
|                 |               |          |                |            |                | AB426949             |
|                 |               |          |                |            |                | AB426950             |
| *P. delicatulus* | Sympatric     | 48       | 1              | 1          | 5              | LC651835             |
|                 |               |          |                |            |                | LC651836             |
|                 |               |          |                |            |                | LC651837             |
|                 |               |          |                |            |                | LC651838             |
|                 |               |          |                |            |                | LC651839             |
| *P. delicatulus* | Sympatric     | 50       | 1              | 1          | 1              | LC651840             |
| *P. delicatulus* | Sympatric     | 51       | 1              | 5          | 3              | 1                    | same as LC651840     | LC651913             |
|                 |               |          |                |            |                | LC651841             |
|                 |               |          |                |            |                | LC651842             |
| *P. delicatulus* | Sympatric     | 54       | 1              | 1          | 2              | 1                    | LC651843             | LC651914             |
|                 |               |          |                |            |                | LC651844             |
| *P. delicatulus* | (Others)      | 58       | 1              | 1          | 1              | AB609384             | LC651915             |
| *P. delicatulus* | (Others)      | 59       |                | 1          |                | LC651845             |
| *P. delicatulus* | (Others)      | 60       | 4              | 1          |                | AB609385             | LC651916             |
|                 |               |          |                |            |                | AB609386             |
|                 |               |          |                |            |                | AB609387             |
|                 |               |          |                |            |                | AB609388             |
| *P. delicatulus* | (Others)      | 64       |                | 2          |                | AB609389             |
|                 |               |          |                |            |                | AB609390             |
| *P. delicatulus* | (Others)      | 65       | 3              | 1          |                | AB426954             | LC651917             |
|                 |               |          |                |            |                | AB609391             |
|                 |               |          |                |            |                | AB609392             |
| *P. delicatulus* | (Others)      | 67       |                | 1          |                | LC651846             |
| *P. delicatulus* | (Others)      | 68       |                | 1          |                | LC651847             |
| *P. delicatulus* | (Others)      | 69       | 1              | 1          |                | LC651848             | LC651918             |
| *P. delicatulus* | (Others)      | 70       | 3              | 1          |                | AB609393             | LC651919             |
|                 |               |          |                |            |                | AB609394             |
| *P. delicatulus* | (Others)      | 71       | 2              | 1          |                | LC651849             | LC510902             |
|                 |               |          |                |            |                | LC651850             |
| *P. delicatulus* | (Others)      | 72       | 3              | 1          |                | AB609396             | LC651920             |
|                 |               |          |                |            |                | AB609397             |
|                 |               |          |                |            |                | AB609398             |
| *P. delicatulus* | (Others)      | 73       |                | 1          |                | AB426955             |
| Species   | Analysis Unit | Site No. | Number examined | Morphology | Genetic region | Accession No. of DDBJ |
|-----------|---------------|---------|----------------|------------|----------------|----------------------|
|           |               |         |                | Male | Female | COI  | ITS  | COI  | ITS  |
| P. delicatulus (Others) | 74          |         | 1               | 1     |        | LC651851 | LC651921 |
| P. delicatulus (Others) | 75          |         | 3               |        |        | AB609401 |          |
| P. delicatulus (Others) | 76          |         | 2               |        |        | AB609400 |          |
| P. delicatulus (Others) | 77          |         | 2               |        |        | AB609400 |          |
| P. delicatulus (Others) | 78          |         | 4               | 2     |        | AB609405 | LC651922 |
| P. delicatulus (Others) | 79          |         | 2               |        |        | AB609408 |          |
| P. delicatulus (Others) | 80          |         | 1               |        |        | AB426957 |          |
| P. delicatulus (Others) | 81          |         | 2               | 1     |        | AB426958 |          |
| P. delicatulus (Others) | 82          |         | 3               | 2     |        | AB426959 | LC651924 |
| P. kawadai No introgression | 83         |         | 1               | 2     | 1     | LC651856 | LC651905 |
| P. kawadai No introgression | 34         |         | 5               | 8     | 2     | LC651857 | LC651926 |
| P. kawadai No introgression | 35         |         | 1               | 1     | 1     | LC651858 |          |
| P. kawadai No introgression | 36         |         | 13              | 5     | 3     | AB426962 |          |
| P. kawadai No introgression | 37         |         | 2               | 2     | 1     | LC651860 | LC651927 |
| P. kawadai No introgression | 84         |         | 3               | 4     |        | LC651861 |          |
| P. kawadai No introgression | 85         |         | 6               | 3     | 4     | LC651862 | LC651928 |
| P. kawadai No introgression | 86         |         | 3               | 3     | 3     | AB426965 | LC651929 |
| P. kawadai No introgression | 87         |         | 2               | 1     | 3     | AB426966 |          |
| P. kawadai No introgression | 88         |         | 2               | 1     | 3     | AB426967 |          |
| P. kawadai No introgression | 48         |         | 1               |        |        | LC651872 |          |
| P. kawadai No introgression | 49         |         | 1               | 1     | 1     | LC651873 | LC651934 |

(Continues)
| Species     | Analysis Unit | Site No. | Number examined | Morphology | Genetic region | Accession No. of DDBJ |
|------------|---------------|----------|----------------|------------|----------------|-----------------------|
|            |               |          |                | Male  | Female | COI | ITS |              |                      |
| P. kawadai | No introgression | 89      | 2              | 2     | 2     | 2   |     | LC651874      |
| P. kawadai | No introgression | 50      | 1              | 1     |       |     |     | LC651876      |
| P. kawadai | No introgression | 90      | 2              | 2     | 1     |     |     | LC651877 LC651935 |
| P. kawadai | No introgression | 91      | 1              | 1     |       |     |     | AB609408      |
| P. kawadai | No introgression | 93      | 1              | 1     | 2     |     |     | AB426968 AB426969 |
| P. kawadai | No introgression | 94      | 10             | 17    | 1     | 1   |     | LC651879 LC651936 |
| P. kawadai | No introgression | 97      | 2              | 3     | 2     | 1   |     | LC651880 LC510906 |
| P. kawadai | Introgression   | 98      | 2              | 3     | 3     | 1   |     | AB609409 LC651937 |
| P. kawadai | Introgression   | 99      | 10             | 6     | 6     | 2   |     | LC651882 LC651883 LC651938 |
| P. kawadai | Introgression   | 100     | 4              | 7     | 2     | 1   |     | LC651889 LC510904 |
| P. kawadai | Introgression   | 44      | 8              | 8     | 2     | 2   |     | LC651890 LC651940 |
| P. kawadai | Introgression   | 105     | 12             | 9     | 2     | 1   |     | LC651893 LC651942 |
| P. kawadai | Introgression   | 106     | 16             | 2     | 2     | 1   |     | LC651894 LC651943 |
| P. kawadai | Introgression   | 107     | 16             | 9     | 2     | 2   |     | LC651895 LC651944 |
| P. kawadai | Introgression   | 111     | 16             | 16    | 3     | 1   |     | LC651899 LC651946 |
| P. kawadai | Introgression   | 112     | 1              |       |       |     |     |                      |
| P. akitaorum |            | 115     | 1              | 1     | 1     |     |     | AB609552 LC510919 |
| P. akitaorum |            | 29      | 1              |       |       |     |     | AB427035      |
| P. akitaorum |            | 116     | 1              |       |       |     |     | AB427039      |
| P. akitaorum |            | 117     | 1              |       |       |     |     | AB609555      |
| P. sugitai |            | 118     | 1              |       |       |     |     | AB588791      |
| P. sugitai |            | 119     | 1              |       |       |     |     | AB588790      |
| P. sugitai |            | 65      | 1              | 1     | 1     |     |     | AB588793 LC510920 |
| P. sugitai |            | 120     | 1              |       |       |     |     | AB588811      |
| P. sugitai |            | 121     | 1              |       |       |     |     | AB609559      |