Incipient allochronic speciation by climatic disruption of the reproductive period

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Disruptive selection of life-cycle timing may cause temporal isolation directly and, ultimately, allochronic speciation. Despite the fact that segregation of the reproductive period among related species has been broadly observed across taxa, it remains controversial whether temporal isolation can function as the primary process of speciation. In the Japanese winter geometrid moth *Inurois punctigera*, allochronic divergence has resulted from climatic disruption of the reproductive period. In habitats with severe midwinter, two sympatric groups of moth reproduce allochronically in early and late winter. These groups are genetically diverging sister lineages and now co-occur allochronically throughout Japan. By contrast, in habitats with milder midwinter these lineages form a continuous adult period and gene flow has been facilitated between the lineages. These results, together with the fact that there is no difference in larval host use, indicate that temporal isolation has been the sole mechanism for allochronic isolation in colder habitats and that allochrony is not a by-product of other adaptations. Thus, the allochronic divergence of sympatric *I. punctigera* populations represents an incipient speciation process driven by midwinter disruption of the reproductive period.

Keywords: seasonal adaptation; temporal isolation; isolation by time; winter geometrid moth

1. INTRODUCTION

The role of adaptation in speciation has been a central issue in evolutionary biology ever since Darwin (1859). Empirical evidence for ecological speciation has accumulated during the last two decades (Nosil *et al.* 2002; McKinnon *et al.* 2004; Ryan *et al.* 2007). Although most empirical studies of ecological speciation have focused on adaptive divergence in food or habitat use and associated reproductive isolation (Schluter 2001; Dieckmann *et al.* 2004; Rundle & Nosil 2005), temporal division of the reproductive period is a common form of prezygotic isolation in many organisms and may also be involved in speciation processes (Coyne & Orr 2004). The term ‘allochronic speciation’ was coined by Alexander & Bigelow (1960) to describe a special case of sympatric speciation, in which temporal isolation leads to speciation without other, preceding isolation mechanisms. Temporal isolation may be involved in speciation processes as a by-product of adaptation to different hosts or to different habitats (Filchak *et al.* 2000; Thomas *et al.* 2003; Hall & Willis 2006), but such cases are excluded from allochronic speciation. Alexander & Bigelow (1960) postulated that, for a pair of field cricket species, disruptive selection for alternative cold-resistant overwintering stages resulted in segregation of the reproductive period and hence speciation occurred between sympatric populations with different seasonal life cycles. Although their example was not valid because the field crickets were not sister species (Huang *et al.* 2000), divergence in seasonal life cycles is a prominent aspect of the life-history evolution of groups

as diverse as insects (Tauber *et al.* 1986), and it may promote speciation by temporal isolation. Previous studies have reported additional cases of putative allochronic speciation in various organisms (Filchak *et al.* 2000; Simon *et al.* 2000; Abbot & Withgot 2004; Friesen *et al.* 2007; Santos *et al.* 2007; Tomaiuolo *et al.* 2007; Devaux & Lande 2008), but the empirical evidence for allochronic speciation is weak and inconclusive because both the disruptive selection force causing temporal isolation and the relative importance of temporal isolation in population divergence remains unclear. Also, it is difficult to determine the geographical mode of population divergence (allopatric, parapatric or sympatric) leading to the temporal isolation. Sympatric populations with temporal isolation can result from a secondary contact of populations, which acquired allochronic life cycles in allopatry (Mayr 1963; Abbot & Withgot 2004).

A novel case of speciation by temporal isolation can be found in a group of winter geometrid moths. These moths emerge and reproduce during winter, typically exhibiting winglessness in females but not in males (figure 1a). This unusual life history has repeatedly evolved within different lineages of this family (Yamamoto & Sota 2007). Although the winter moths are adapted to cold, their emergence and activity are prevented by excessively low temperatures and deep snow. Therefore, in habitats with unfavourable midwinters, the period of adult emergence and reproduction (‘flight period’, for simplicity) can be shifted towards early or late winter or even disrupted into both. This phenological variation is known for the genus *Inurois* of the winter moth subfamily Alsophilinae (Nakajima 1998). In *Inurois punctigera*, the flight period of adults is disrupted in Japanese habitats with colder midwinters, although it is continuous in habitats with mild

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Figure 1. Geometrid winter moths and their life-history variation along a climatic gradient. (a) Copulating *Inurois tenuis* male and female. (b) Seasonal life cycles of *Inurois punctigera* at (i) continuous flight period site and (ii) disrupted flight period site (modified from Nakajima 1998). (c, d) Collection date of moths plotted against the mean temperature of the coldest month at the collection site for (c) *I. punctigera* and (d) other *Inurois* species in Japan. Orange diamonds, *I. nikkoensis*; green diamonds, *I. kobayashii*; red diamonds, *I. kyushuensis*; orange pluses, *I. asahinai*; green pluses, *I. fumosa*; red pluses, *I. fletcheri*; and blue pluses, *I. tenuis*.

winters (figure 1b). Adult longevity is only approximately two weeks, and the moths occurring in early and late winter at the same place represent different cohorts

(Nakajima 1998). *Inurois* larvae are generalist feeders on deciduous broadleaf trees, and larvae of early- and late-winter moths hatch simultaneously in the spring and pupate by early summer (Nakajima 1998). Therefore, early- and late-winter cohorts differ in the timing of their seasonal life cycles but not in host use by the larvae. The divergence in the flight period of *Inurois* is presumably based on genetic divergence in the length of the pupal period, because the timing of the life history of insects with annual life cycles is controlled by genetically-based responses to seasonal conditions (Tauber *et al.* 1986). Indeed, evidence exists for genetic control of the length of the pupal period in geometrid moths (Tammaru *et al.* 1999). Thus, life cycle divergence in *I. punctigera* may represent a case of incipient isolation due to the temporal isolation induced by midwinter cold.

In this study, we examined whether the temporal isolation, resulting from midwinter disruption, of *I. punctigera* adheres to the model of the allochronic speciation process. Using mitochondrial and nuclear DNA markers, we found that: (i) the allochronic populations are sister taxa; (ii) genetic divergence had occurred between them; and (iii) temporal isolation is the primary cause of reproductive isolation. One approach to the last point is to demonstrate that allochronic populations fuse when the temporal isolation factor (winter cold) is removed (Coyne & Orr 2004). We show that, along a climatic gradient, the fusion of allochronic populations has occurred at a site with mild winters but not at sites with cold winters.

2. MATERIAL AND METHODS

(a) Data collection, sampling and field study

The geometrid genus *Inurois* occurs in the Russian Far East, Northeast China, the Korean Peninsula and Japan, where it is more species-rich (e.g. Beljaev 1996). Adult periods of *Inurois* across Japanese islands (figure 1c,d) were examined based on the literature and the authors' collection data (see text S1 and table S1 in the electronic supplementary material). The mean temperature of the coldest month at each collection site used in figure 1c,d was collected from the Mesh Climate Data 2000 (Japan Meteorological Agency), which compiles data from 1971 to 2000. For DNA studies, we collected 782 *I. punctigera* moths from 16 sites throughout Japan and 85 specimens of seven other species of *Inurois* occurring in Japan (see table S1 in the electronic supplementary material). Detailed analysis of gene flow between seasonal cohorts of *I. punctigera* were conducted at four main study sites in Honshu: Sendai (indicated as SD in figures 2–4; 38°16'22" N, 140°32'53" E; annual mean temperature, $T_m = 9.3^\circ\text{C}$; mean temperature of coldest month, $T_c = -2.1^\circ\text{C}$); Kobuchizawa (KB; 35°52'25" N, 138°19'32" E; $T_m = 9.6^\circ\text{C}$; $T_c = -1.9^\circ\text{C}$); Gifu (GF; 35°25'33" N, 136°46'09" E; $T_m = 14.5^\circ\text{C}$; $T_c = 3.2^\circ\text{C}$); and Kyoto (KY; 35°02'10" N, 135°47'52" E; $T_m = 14.5^\circ\text{C}$; $T_c = 3.3^\circ\text{C}$). Sendai and Kobuchizawa are high-latitude and high-altitude sites, respectively, with disrupted flight periods separated by two to three months. Gifu is a low-altitude site located at the margin of the central mountain range, with disrupted flight periods separated by approximately three weeks. Kyoto is a low-altitude site with a continuous flight period. At Gifu and Kyoto, we conducted weekly collection during the 2007–2008 winter to study seasonal prevalence of adults and genetic differentiation between weekly cohorts.

Figure 2. Mitochondrial and nuclear gene trees of *Inurois*. (a) Mitochondrial COI gene. Red represents the early-winter group and blue represents the late-winter group. The trees are neighbour-joining trees. Node supports are bootstrap percentages in NJ and ML analyses followed by Bayesian posterior probability (shown when > 70% for NJ). (b) Nuclear elongation factor 1α. For *I. punctigera*, collection site (SD, Sendai; KB, Kobuchizawa; GF, Gifu; KY, Kyoto), flight period (red, early winter; blue, late winter; grey, continuous flight period site), and mitochondrial clades (yellow, A; green, B) of the specimen are indicated.

On each census day, we searched flying males for 1 hour starting 30 min after sunset (see table S1 in the electronic supplementary material).

(b) Laboratory protocols

DNA extraction was performed using DNA Purification Kit (Promega). A portion of mitochondrial cytochrome oxidase subunit I (COI) gene region was PCR-amplified using primers 5'-TTA TTT TTG GAA TTT GAG C-3' (forward) and 5'-CCT GTT AAT CCT CCT ACT GT-3' (reverse),

and a 518-bp fragment was sequenced using the forward primer. In addition, a 934-bp fragment of nuclear elongation factor-1α (EF-1α) gene region was PCR-amplified using primers 5'-TGC GGT GGT ATC GAC AAG AG-3' and 5'-GAT TTA CCR GWA CGA CGR TC-3' (Kawakita *et al.* 2004). The PCR products were sequenced using an ABI3130xl sequencer (Applied Biosystems). Sequence data have been deposited in GenBank (accession numbers: AB467868–AB468050). Amplified fragment length polymorphism (AFLP) analysis (Vos *et al.* 1995) was performed

Figure 3. (a) Statistical parsimony tree with geographical distribution of haplotypes. The size of each haplotype reflects the number of samples, and the pie graph indicates the proportions of individuals from different regions distinguished by colours as in (b). Internal haplotypes immediately on both sides of the connection path of clades A and B are encircled by a broken line. (b) Sampling areas in Japan. Locality codes are those used in the electronic supplementary material, table S2, which gives exact distributions of haplotypes. Filled circles, continuous flight period site; open circles, disrupted flight period site.

for *I. punctigera* specimens collected at the four main study sites during the 2007–2008 winter. We used a plant mapping kit (Applied Biosystems) and four primer combinations for selective amplification (EcoRI+/MseI+: ACA/CTG, ACT/CTG, ACG/CAA, AAC/CAC). The amplified fragments were electrophoresed on an ABI 3130xl sequencer and binary-coded using GENE-MAPPER v. 4.0 (Applied Biosystems). To ensure high reliability of analysed AFLP loci, every moth was genotyped twice (Pompanon *et al.* 2005), and a total of 676 loci with more than 95 per cent repeatability were used in the analysis.

(c) Phylogenetic and statistical analyses

Phylogenetic trees of COI and EF-1 α sequences were constructed by the neighbour-joining method with Tamura-Nei (COI) and GTR (EF-1 α) substitution model using PAUP* v. 4.10b (Swofford 2002), a Bayesian inference method with GTR+I+G substitution model using MrBAYES

v. 3.12 (Huelsenbeck & Ronquist 2001), and a maximum likelihood (ML) method with GTR+I+G substitution model using GARLI v. 0.96 (<http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html>). The substitution models used in the Bayesian and ML methods were determined by MrMODELTEST v. 2.2 (Nylander 2004) and MODELTEST v. 3.7 (Posada & Crandall 1998). The reliability of nodes was evaluated by 1000 bootstrap replications in the NJ and ML methods and posterior probabilities in the Bayesian method. To infer the geographical divergence process of COI haplotypes, a statistical parsimony network was constructed using TCS v. 1.21 (Clement *et al.* 2000); loops in the network were resolved following Pfenninger & Posada (2002). The nested clade phylogeographic analysis (Templeton 2004) was performed using ANECA v. 1.1 (Panchal 2007). For the four main study sites, genetic differentiation between allochronic or allopatric populations was evaluated with the fixation index F_{st} using ARLEQUIN v. 3.11 (Excoffier *et al.* 2005) for COI and AFLP-SURV v. 1 (Vekemans *et al.* 2002) for AFLP data. To reveal the genetic differentiations among study sites and between early- and late-winter populations for three study sites with sympatric allochronic populations, we performed an analysis of molecular variance (AMOVA) for both COI and AFLP data using ARLEQUIN. The relationships among populations were depicted by neighbour-joining trees based on Nei's genetic distance (Nei & Li 1979). To assess the reliability of nodes, we performed bootstrap analyses. For COI data, genetic distances were obtained by ARLEQUIN, bootstrap data (100 replications) were created using EXCEL, and the tree was drawn by PHYLIP v. 3.6 (Felsenstein 1989). For AFLP data, AFLP-SURV was used for all the analyses (bootstrapping: 999 replications). Variation in the AFLP composition of individual moths was evaluated by a principal coordinate (PCO) analysis implemented in GENEALX v. 6.1 (Peakall & Smouse 2006). To determine whether the incongruence of mitochondrial lineage with flight period was also found for nuclear genomes, a canonical discriminant analysis of AFLP genotypes was performed for moths with different emergence periods and mitochondrial lineage at three sites with disrupted flight periods using JMP v. 5.0.1j (SAS Institute, Inc.). Scores of six PCOs for individual moths obtained by the PCO analysis of AFLP data were used in this analysis. The association between time lag of moth activity and genetic difference was examined for specimens collected weekly at Gifu and Kyoto using a Mantel test implemented in ARLEQUIN. We considered each weekly cohort as a temporal population and calculated pairwise F_{st} between weekly cohorts. The first and eighth week at Gifu were not included because we collected only one specimen in these weeks (see table S1 in the electronic supplementary material).

3. RESULTS

(a) Flight period phenology along a climatic gradient

The flight period of *I. punctigera* was disrupted in colder habitats, whereas it was continuous in habitats with milder winters, as defined by the mean temperature of the coldest month (figure 1c). In seven other *Inurois* species occurring in Japan, the same overall pattern of flight period divergence was observed, although each species exhibited either early- or late-winter flight (figure 1d). Only *Inurois tenuis* exhibited late-winter flight, while other species exhibited early-winter flight. A single record exists

Figure 4. Geographic pattern of genetic differentiation between six allochronic populations at three disrupted flight period sites and a population at continuous flight period site of *I. punctigera*. (a) Four study sites (open circles) and geographic pattern of winter coldness in Japan. Insets show the seasonal prevalence of *I. punctigera* moths at each site. For Sendai and Kobuchizawa, flight periods are indicated by bars based on collection records in the 2007–2008 winter. For Gifu and Kyoto, the number of males collected every 1 hour is shown based on weekly census during the 2007–2008 winter. Mean temperature of the coldest month: blue, below 0°C; yellow, 0–3°C; pink, above 3°C. (b, c) Population trees based on (b) mitochondrial COI gene sequence and (c) amplified fragment length polymorphisms. The unrooted trees were constructed based on Nei's genetic distance between populations. Node supports are bootstrap values (shown when > 50%). Sample sizes and haplotype diversity (b) or average heterozygosity (c) are indicated for each population. Genetic divergences between sympatric allochronic populations are indicated by F_{st} values (** $p < 0.001$).

of *Inurois asahinai* in late winter, but the late-winter occurrence of this species was not observed thereafter (see Nakajima 1998).

(b) Phylogenetic and geographic origin of early- and late-winter moths

The monophyletic origin of *I. punctigera* moths with different flight periods was demonstrated by the molecular phylogenies of the genes encoding mitochondrial COI (figure 2a) and nuclear EF-1 α (figure 2b). The phylogenetic trees indicated that all *I. punctigera* haplotypes are closely related to one another and distantly related to other *Inurois* species. The mitochondrial lineage of *I. punctigera* consisted of two clades, A and B, which corresponded to the two major clades in the statistical parsimony

network (figure 3a; see fig. S1 in the electronic supplementary material). Most (82%) early winter moths possessed clade A haplotypes, and most (98%) late-winter moths had clade B haplotypes. Moths from continuous flight period sites were included in both clades. Thus, clades A and B primarily consisted of early- and late-winter moths, respectively.

In the nested-clade phylogeographic analysis, clades A and B were inferred to have undergone range expansions (see table S3 in the electronic supplementary material). The internal haplotypes located immediately on both sides of the connection path occurred in southwest Honshu and Shikoku, and the putatively derived haplotypes occurred in central to northern Honshu, Hokkaido and Kyushu (figure 3a,b).

Figure 5. Genetic difference among individual moths in different allochronic cohorts at four study sites. Genetic difference is expressed by first and second PCO scores based on AFLPs. Differences in flight date are indicated by different colours and symbols. (a–c) Disrupted flight period sites: (a) Sendai (red diamonds, early; blue diamonds, late); (b) Kobuchizawa (red diamonds, early; blue diamonds, late); (c) Gifu (red diamonds, 10 December; red triangles, 16 December; red circles, 21 December; red squares, 29 December; pink diamonds, 4 January; blue diamonds, 26 January; blue triangles, 1 February; blue circles, 8 February; blue squares, 15 February; light blue diamonds, 22 February; light blue triangles, 29 February). (d) Continuous flight period site: Kyoto (red diamonds, 31 December; red triangles, 7 January; red circles, 14 January; red squares, 21 January; pink diamonds, 27 January; blue diamonds, 4 February; blue triangles, 18 February; blue circles, 18 February; blue squares, 25 February; light blue diamonds, 3 March; light blue triangles, 10 March).

Figure 6. Relationship between difference in flight period and genetic isolation among weekly cohorts of *I. punctigera*. (a) Disrupted flight period site (Gifu). Closed circles indicate F_{st} for weekly cohort pairs within the same flight periods, and open circles represent pairs between the two flight periods. (b) Continuous flight period site (Kyoto). Molecular markers are (i) AFLPs and (ii) mitochondrial COI haplotypes.

(c) Genetic divergence between moths with different flight periods

The genetic divergence between sympatric moths with different flight periods was studied at four representative sites along the climatic gradient (figure 4a) using mitochondrial COI gene sequences and AFLPs. For both genetic markers, AMOVA showed that there was

significant differentiation between early- and late-winter moths (F_{sc}) but not among the three disrupted flight period sites (F_{ct} ; COI: $F_{sc}=0.549$, d.f.=3, $p<0.001$; $F_{ct}=-0.113$, d.f.=2, $p=0.659$; AFLP: $F_{sc}=0.0659$, d.f.=3, $p<0.001$; $F_{ct}=0.0146$, d.f.=2, $p=0.0763$). The pairwise F_{st} value between early- and late-winter moths was always significantly greater than 0 (figure 4b,c).

The population tree based on the mitochondrial gene had high nodal supports and revealed that early- and late-winter moth populations form different lineages that are connected to the continuous flight period site population at Kyoto (figure 4b). According to the AFLP data, geographical differentiation occurred between northern (Sendai, Kobuchizawa) and southern (Gifu, Kyoto) sites. At the northern sites, populations with the same flight periods were grouped together, although the divergence between groups was small and not well-supported (figure 4c).

PCO analysis of AFLPs demonstrated that genetic divergence of individual moths between early- and late-winter populations was evident at the disrupted flight period sites (figure 5a–c); however, among moths appearing in different weeks, genetic divergence was unclear at the continuous flight period site in Kyoto (figure 5d). Some individuals from the disrupted flight period sites exhibited inconsistency between flight period and mitochondrial lineage (figure 2a). However, a canonical discriminant analysis of AFLP genotypes revealed that these individuals could be classified into groups that were consistent with their flight periods (see table S4 in the electronic supplementary material).

Finally, the genetic compositions of weekly cohorts at a disrupted flight period site and a continuous flight period site (Gifu and Kyoto, respectively) were compared by plotting F_{st} values against time lags (figure 6). At Gifu, F_{st} within the same flight periods ($\text{lag} \leq 4$ weeks) was effectively 0, while F_{st} between the disrupted flight periods ($\text{lag} \geq 4$ weeks) was much greater than 0 (figure 6a). At Kyoto, genetic differentiation among moths flying in different weeks was gradual, and for both DNA markers there were positive correlations of F_{st} and time lag between weekly cohorts (i.e. isolation-by-time; Hendry & Day 2005; figure 6b; Mantel test; AFLP: $r = 0.286$, $p = 0.033$; COI: $r = 0.577$, $p = 0.001$).

4. DISCUSSION

The monophyletic origin of *I. punctigera* moths with different flight periods was demonstrated by molecular phylogenetic analyses of mitochondrial and nuclear gene sequences (figure 2). The phylogenetic trees also revealed another divergence of early- and late-winter moths in *Inurois*, namely *I. fumosa* and *I. tenuis*, which are clearly discriminated as different species, indicating repeated divergence of flight periods within this genus. The highly biased distributions of early- and late-winter moths between mitochondrial clades A and B of *I. punctigera* suggested that these clades diverged in association with the divergence of flight periods. Moths from continuous flight period sites possessed haplotypes of both clades, suggesting secondary contacts of the once-separated early- and late-winter populations. Each of the clade A and B haplotypes was inferred to have undergone range expansion. Based on the assumption that interior haplotypes are ancestral relative to tip haplotypes (e.g. Pfenninger & Posada 2002), the internal haplotypes immediately on both sides of the connection path in the COI haplotype network probably indicate the source area of these range expansions. The internal haplotypes occurred mainly in southwest Honshu and Shikoku; populations in these areas possessed the internal haplotypes almost exclusively, whereas derived haplotypes occurred mostly in central and

northern Honshu, Hokkaido and Kyushu. Thus, the lineages of early- and late-winter moths probably originated from the region of southwest Honshu and Shikoku, and both moths expanded their ranges over the Japanese islands without extensive gene flow between them. As the present *I. punctigera* populations in the putative source areas exhibit a continuous flight period, the initial divergence of early- and late-winter moths probably occurred during one of the glacial periods, when southwest Japan had much colder winters. After the initial divergence, moths with different flight periods expanded their ranges during the post-glacial period, whereas in areas with milder winters owing to post-glacial warming, the diverged flight periods fused as a result of diminished midwinter disruptive selection. Thus, continuous flight periods are exhibited in areas with milder winters, whereas disrupted flight periods have been maintained in areas with colder winters, resulting in the characteristic 'inverse V' profile of moth occurrence along the climatic gradient (figure 1c,d). However, a more detailed study is needed to examine our historical biogeographic hypothesis. Owing to the limited phylogeographic data and the lack of appropriate statistical analyses, we cannot determine whether the initial divergence of early- and late-winter moths was strictly sympatric. However, the most important point remains that the initial divergence was probably caused by the common, disruptive selection force within an area and not by local adaptations to different climatic conditions, because different local adaptations would preclude the two life cycles from co-occurring over a broad range of climatic conditions. Therefore, the alternative hypothesis of a purely allopatric origin of allochrony with different local adaptations (e.g. Mayr 1963; Abbot & Withgott 2004) can be excluded in our case.

Significant genetic divergence occurred between allochronic populations at the disrupted flight period sites (Sendai, Kobuchizawa, Gifu). By contrast, at the continuous flight period site (Kyoto), the genetic divergence among weekly cohorts was gradual, as indicated by weak trends of 'isolation-by-time' (figure 6b). These results suggest that the moths with different flight periods are isolated only allochronically, due to midwinter disruption, but otherwise can interbreed in the absence of substantial reproductive isolation. At the same time, the genetic divergence between early- and late-winter moths at the disrupted flight period sites and among weekly cohorts in the continuous flight period site implies that the timing of adult emergence is genetically determined. In addition, the isolation-by-time pattern at the continuous flight period site indicates that the scale of the genetic difference in the timing of adult emergence is as fine as weekly. Some individuals from disrupted flight period sites exhibited inconsistency between flight period and mitochondrial lineage (figure 2a), but their nuclear genotypes could be classified into groups that were consistent with their flight periods (see table S4 in the electronic supplementary material). The incongruence of mitochondrial lineage and phenotype may have resulted from occasional shifts of emergence period (and hybridization), but the introgression of nuclear genes has not been substantial.

The ongoing allochronic divergence of *I. punctigera* in habitats with severe winters may represent the incipient phase of allochronic speciation. The sister pair of the early- and late-winter species *I. fumosa* and *I. tenuis* is

suggestive of the possibility of the allochronic speciation in this genus. Future studies of the differentiation processes of these species will help us to understand the speciation process with respect to divergent flight periods. For *I. punctigera* to complete speciation, temporal isolation must last long enough that it causes the evolution of other isolating mechanisms, such as different sex pheromones and post-zygotic incompatibility. Genetic differentiation by temporal isolation at disrupted flight period sites can be hindered by gene flow from continuous flight period sites. For example, in AFLP genotypes, the early- and late-winter populations at Gifu were not directly related to those at the other disrupted flight period sites, but both were related to the Kyoto population (figure 4c); this may be due to gene flow between disrupted and continuous flight period sites. However, the dispersal power of *Inurois* is limited; females are flightless and, although males fly weakly and young larvae disperse by ballooning (Sakanoshita 1997), these stages are unlikely to succeed in long-distance dispersal. Thus, in high-latitude or high-altitude areas with few continuous flight period sites, gene flow from such sites would be fairly restricted, resulting in a greater chance that temporal isolation is maintained for a long time.

Allochronic speciation may involve two different patterns: one caused by seasonal segregation, as described in this and several other studies (Abbot & Withgot 2004; Friesen *et al.* 2007; Santos *et al.* 2007); and another caused by segregation between years, as in periodical cicadas (Simon *et al.* 2000). Whereas the latter case is certainly rare (Coyne & Orr 2004), allochronic divergence by seasonal segregation may be a more common pattern of temporal isolation in organisms living in a seasonal environment, because almost all organisms must cope with the seasonal harshness of the abiotic environment, including coldness and desiccation. Our study elucidates the pivotal role of seasonally disruptive selection and the resultant allochronic divergence in organismal diversity.

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