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Phylogenetic analysis of the winter geometrid genus *Inurois* reveals repeated reproductive season shifts ☆

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ABSTRACT

Winter geometrid moths belonging to the genus *Inurois* comprise nine species that reproduce during early winter, three species that reproduce in late winter, and polymorphic species with genetically diverged early and late winter populations that co-occur widely across the species' range. In our previous studies, we demonstrated that differences in reproductive timing resulted in allochronic reproductive isolation between sympatric populations. In the present study, to assess the evolutionary pattern of reproductive timing within the genus, we determined the phylogenetic relationships among species using nuclear and mitochondrial gene sequences. Nuclear gene tree showed that reproductive season shifts occurred independently in four of 13 divergence events. In two divergence events, allochronic sister lineages were formed in sympatry, suggesting that the segregation of the reproductive season was associated with diversification in the genus *Inurois*. We also found that the mitochondrial gene tree was quite different from the nuclear gene tree and that mitochondrial introgression may have occurred in a few cases. Although it remains unclear whether early and late winter species actually have hybridized with each other and how strong or stable is the reproductive isolation provided by the reproductive season segregation, our study illuminates the potential importance of allochronic isolation in the diversification process of the genus *Inurois*.

1. Introduction

Temporal disjunction of reproductive activities functions as a hybridization barrier between closely related species (Lamont et al., 2003; Danley et al., 2007; Sachet et al., 2009; Thomassen et al., 2013) as well as facilitates genetic differentiation among populations (Hall and Willis, 2006; Smith and Friesen, 2007; Sota et al., 2013) and sexual isolation among individuals within populations (Hirao and Kudo, 2008). Reproductive isolation owing to differences in reproductive timing is known as allochronic isolation. This isolation should be important for understanding the mechanism of diversification in a specific lineage, because in some cases, allochronic isolation can be an initial step of speciation (Alexander and Bigelow, 1960; Marshall and Cooley, 2000; Abbot and Withgott, 2004; Friesen et al., 2007; Yamamoto and Sota, 2009; Santos et al., 2007).

Allochronic isolation is often strongly associated with divergence in ecological traits, such as host preferences in phytophagous insects (Filchak et al., 2000; Berlocher and Feder, 2002; Drès and Mallet, 2002; Thomas et al., 2003; Savolainen et al., 2006; Franks and Weis, 2009; Shimono et al., 2009; Matsubayashi and Katakura, 2009; Papadopulos et al., 2013; Richter-Boix et al., 2013; Thomassen et al., 2013; Powell et al., 2014). In addition, climate can be a major factor that causes allochronic isolation. For example, a seabird species has sympatric populations that breed in different seasons (hot and cool seasons) on separate islands (Monteiro and Furness, 1998; Friesen et al., 2007; Bolton et al., 2008). Another example is the winter geometrid moth *Inurois punctigera* showing a reproductive season that is separated into early and late winter in cool habitats owing to harsh midwinter conditions; however, its reproductive season is not separated in warm habitats (Nakajima, 1998). Genetic differentiation between sympatric early and late winter populations has been demonstrated in habitats with severe midwinter conditions (Yamamoto and Sota, 2009). In these studies, the segregation of reproductive seasons was shown to have occurred in parallel in different localities with similar climatic conditions (Friesen et al., 2007; Yamamoto and Sota, 2012). Given that specific climatic conditions can facilitate allochronic isolation at different localities in a

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similar manner to these cases, the species diversity of a lineage that occupies a wide area, including habitats with similar climatic conditions, may be associated with repeated evolutionary shifts in the reproductive season.

In East Asia, the winter moth genus *Inurois*, including *I. punctigera*, provides an opportunity to examine the hypothesis of repeated reproductive season shifts. This genus exhibits variations in its reproductive season, where some species reproduce in early winter and others in late winter if their habitats have severe midwinter conditions (Fig. 1), suggesting that the separation of reproductive season may have occurred in other lineages of this genus, in addition to *I. punctigera*. Furthermore, given that the divergence of the reproductive season can occur repeatedly in *I. punctigera*, the reproductive season may also have diverged independently in the entire *Inurois* lineage. Because *Inurois* species occupy almost the same geographic range as *I. punctigera*, this genus should have also been affected by the same climatic environment; thus, harsh climatic conditions may have promoted repeated reproductive season shifts in the genus *Inurois*.

In this study, we addressed the evolution of reproductive season in the winter moth genus *Inurois* and the importance of the separated reproductive season in diversification (i.e. speciation and maintenance of reproductive isolation between species). To this end, we studied the phylogenetic relationships among all *Inurois* species using both nuclear and mitochondrial genes. Introgression of mitochondrial gene among Inurois species was also examined. We hypothesized that reproductive season shift has occurred repeatedly in *Inurois* and the separation of reproductive season between species and lineages would play an important role in diversification of Inurois, as suggested in *I. punctigera*.

2. Materials and methods

2.1. Sampling and phylogenetic analyses

Inurois species are distributed around the Sea of Japan (southern Russian Far East, China, Korean Peninsula, and Japan; Fig. 2). We collected fresh specimens to extract DNA from all *Inurois* species in Russia (around Vladivostok) and Japan (Fig. 2, Table 1; see Table S1 for our taxonomic treatment of *Inurois* species). Both early and late winter *I. punctigera* populations were collected. As outgroup taxa, we collected *Alsophiloides acroama* and *Alsophila japonensis* in Japan and *Chimaphila zabolne* in Russia (Table 1). The suitability of these outgroup taxa with respect to *Inurois* was shown in our previous study (Yamamoto and Sota, 2007).

All the specimens were soaked in absolute ethanol and stored in a freezer until DNA extraction. Total genomic DNA was extracted from the flight muscles using a DNA purification kit (Promega, Madison, WI). Partial sequences of three nuclear genes, that is, elongation factor 1 alpha (*EF-1a*), tektin, and arginine kinase (*ArgK*), were polymerase chain reaction (PCR) amplified using the primers 5′-TGCGGTGGTATCGACAAGAG-3′ (Yamamoto and Sota, 2009) and 5′-GATTTACCRGWACGACGRTC-3′ (Kawakita et al., 2004) for *EF-1a*, 5′-ACCAGTGGRGAYATYCTWGG-3′ and 5′-CGCAGTTTYTGATRC TYT-3′ (Mallarino et al., 2005) for tektin, and 5′-GACAG CAARTCTCTGCTGAAGAA-3′ and 5′-GGTYTTGGCATCGTTGTGGTAGA TAC-3′ (Kawakita et al., 2003) for *ArgK*. In addition to these nuclear sequences, a partial sequence of the mitochondrial cytochrome oxidase subunit I (COI) gene was also PCR amplified using the primers: 5′-TTATTTTTGGAATTTGAGC-3′ and 5′-CCTGTTAATCCTACTGT-3′ (Yamamoto and Sota, 2009). A BigDye Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used to analyze the PCR products with an ABI3130xl sequencer (Applied Biosystems) and the same primers. We found that the *ArgK* sequence could not be determined for some samples due to length polymorphisms, so we conducted additional sequencing with the inner forward primer ARGinF 5′-TTCGAGAACTTGGACTCCGG-3′ using the initial PCR products of *ArgK*. Finally, we obtained a 926 bp sequence of *EF-1a*, a 702 bp sequence of tektin, a 415 bp sequence of *ArgK*, and a 900 bp sequence of COI. The sequence data were deposited in GenBank (accession numbers: AB552989–AB553279; AB980810–AB980945). In the molecular phylogenetic analyses, we also used 21 *EF-1a* sequences, which were reported in our previous study (GenBank accession numbers: AB467986–88, AB467990, AB468003–04, AB468006–07, AB468015, AB468028, AB468031, AB468033–35, AB468037–39, AB468044, AB468047–48, AB468050; Yamamoto and Sota, 2009). Genital character states were determined for all the *Inurois* species, as well as *A. japonensis* and *C. zabolne* (Table S2). We could not obtain morphological and nuclear sequence data for *A. acroama*, so the outgroup species were not included in the analyses based on nuclear sequences and morphological data.

Molecular phylogenetic analyses were performed with the three datasets: nuclear sequences (three regions combined), COI sequences, and combined nuclear + morphological data. The sequences of each gene were separated based on the codon positions, and general time reversible (GTR) + G substitution models were applied to each of them. GTR + G substitution models were also applied to the morphological data. The maximum-likelihood (ML) method was performed using RAxML version 8.0.2 (Stamatakis, 2014), and the credibility of each node was evaluated by bootstrap analysis with 1000 replicates. The Bayesian inference method was performed using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001). A partitioned mixed-model analysis was applied, and all model parameter values were "unlinked" among partitions in this analysis. MrBayes was implemented with "diagnfreq" = 3000 and "stopval" = 0.005. The reliability of each node was evaluated based on the posterior probability in the Bayesian analysis.

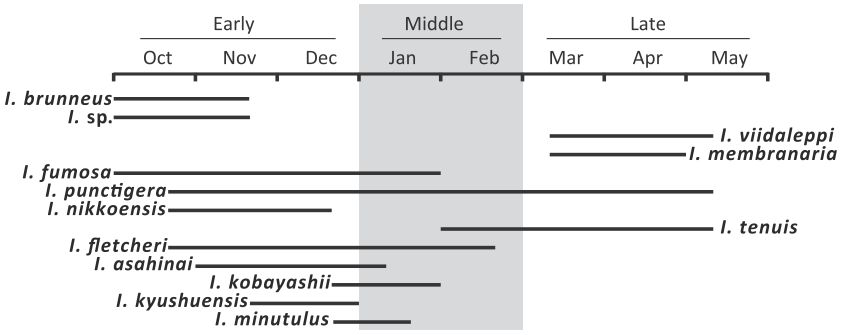

Fig. 1. Flight periods of *Inurois* species. Each bar represents the entire flight period throughout the distribution range of each species. Species are arranged according to their distributions, from northern (i.e., cooler) to southern (i.e., warmer) regions, as described in Table 1.

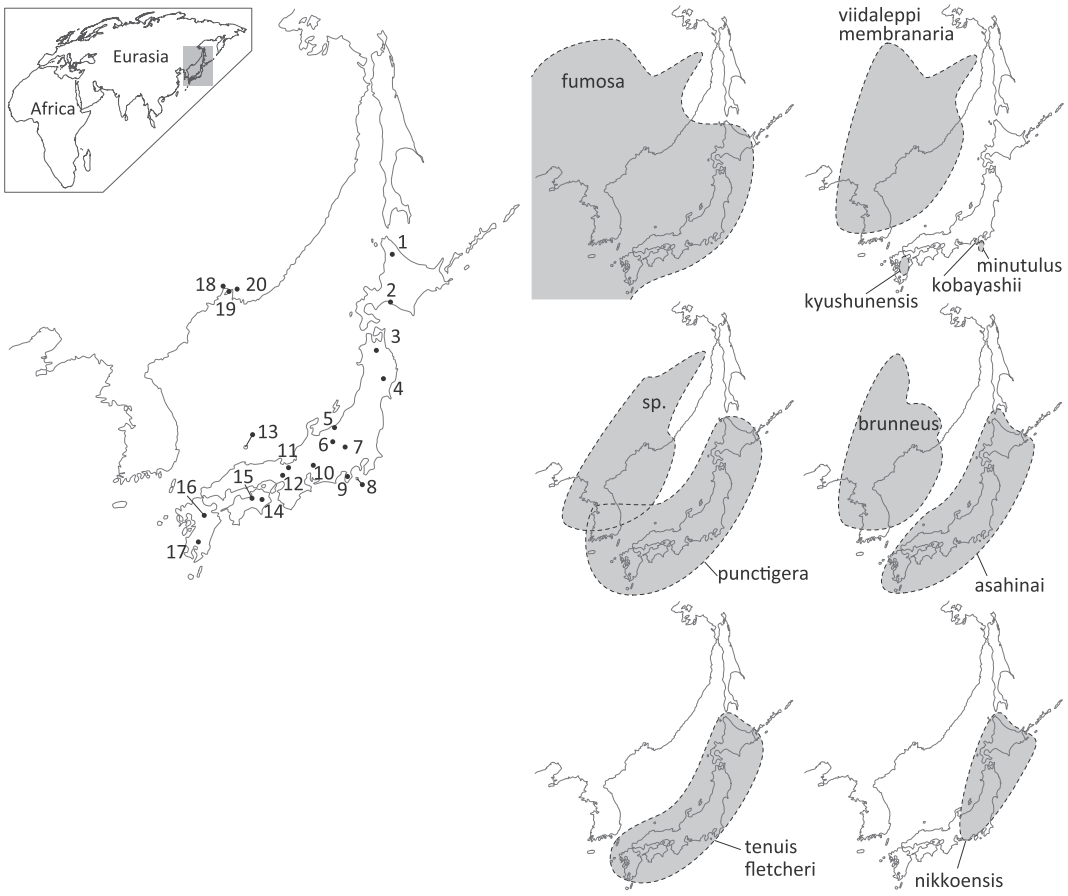

Fig. 2. Distribution range (gray) and the sampling sites of the genus *Inurois*. Note that the presence or absence of *Inurois* on small islands was unclear. Their distribution in eastern part of China is also tentative. The sampling site numbers on the map correspond to those in Table 1.

Table 1
Species analyzed in this study.

Species	N[1]	Collection sites[2]
Inurois brunneus	6	18
Inurois sp.	5	18
Inurois viidaleppi	4	19
Inurois membranaria	8	19
Inurois fumosa	31	1, 2, 6, 7, 12, 13, 14, 16, 18
Inurois punctigera	19	4, 10, 12
Inurois nikkoensis	6	2, 7
Inurois tenuis	8	3, 4, 5, 7, 10, 12, 16, 17
Inurois fletcheri	9	2, 7, 9, 10, 11, 12
Inurois asahinai	9	2, 3, 4, 6, 7
Inurois kobayashii	2	9
Inurois kyushuensis	3	16
Inurois minutulus	6	8
Alsophila japonensis	6	7, 12, 15
Alsophiloides acroama	1	15
Chimaphila zabolne	1	20

[1] Number of specimens.
[2] Collection site numbers corresponds to those shown in Fig. 2.

To examine the certainty of the phylogenetic position of *I. membranaria* suggested based on nuclear gene sequences, we used the approximately unbiased test (AU test; Shimodaira, 2002). We compared the best ML tree, in which *I. membranaria* is sister to the clade including *I. fletcheri* and *I. asahinai*, with an alternative ML tree resulting from ML tree search with the constraint that the clade including *I. punctigera*, *I* sp., and *I. burunneus* is sister to the clade including *I. fletcheri* and *I. asahinai*. Given that the best ML tree suggested that clade including *I. punctigera*, *I* sp., and *I. burunneus* is sister to the clade including I. membranaria, *fletcheri*/*asahinai* groups, this constraint means that *I. membranaria* is not sister to the clade including *I. fletcheri* and *I. asahinai*.

2.2. Analysis of mitochondrial introgression

We found that the mitochondrial and nuclear genealogies were incongruent, and thus, we tested the hypothesis of mitochondrial introgression between *Inurois* species using the program JML version 1.02, which implements Joly's method (Joly et al., 2009; Joly, 2011). To test for mitochondrial gene introgression, the null distribution of the genetic distance between species was calculated based on a coalescent simulation using the combined nuclear dataset, which was assumed to comprise accurate phylogenetic information. Next, we tested the deviation of the mitochondrial genetic distance between species against the null distribution of the genetic distance between species for each pair of species.

To consider the phylogenetic uncertainty of the *Inurois* genus, we constructed 8000 species trees where the estimated population sizes were obtained from 50 million iterations of Bayesian Markov Chain Monte Carlo (MCMC) using the program *BEAST (Heled and Drummond, 2010), which performs Bayesian inference of species-level trees based on coalescent theory. The sequences of the three nuclear genes were partitioned according to their codon positions, and a GTR + G substitution model and exponential relaxed clock (uncorrelated) model were applied to each of the nine partitions. Trees were obtained every 5000 iterations after a burn-in of 10 million iterations. In this procedure, *BEAST in BEAST version 1.8.0 (Drummond and Rambaut, 2007) was used according to the JML manual. We confirmed the convergence of the sampled values

using Tracer version 1.6 (Rambaut et al., 2013). Prior to the JML analysis, we calculated the clock rate of the COI sequence relative to the nuclear sequence, the population size of each lineage, base frequencies, site-specific heterogeneity, and the proportion of invariable sites using BEAST v1.8.0. As a result, the mean relative clock rate of the COI sequence was 5.94 (95% credible interval, 3.79–8.45). We repeated the test for mitochondrial introgression using the mean (5.94) and the lower and upper boundary values (3.79 and 8.45) of the 95% credible interval as the relative substitution rate in the JML analysis. We used the Benjamini–Hochberg method (Benjamini and Hochberg, 1995) to control for the false discovery rate (FDR) and to determine significantly short distances (mitochondrial introgression) at $\alpha = 0.05$ because the JML analysis comprised as many as 1891 comparisons.

2.3. Estimation of ancestral reproductive season

Severe winter conditions may prevent the eclosion of adults, which means that the flight periods of each *Inurois* species differ in warmer and colder habitats (Nakajima, 1998). In warm habitats, the adults of *Inurois* species generally emerge between January and February, which are typically the coldest months of year. In cool habitats, the moths do not emerge in the coldest month, but instead they emerge either before or after the coldest period (note that only *I. punctigera* emerges during the early and late winter in cool habitats). Therefore, although species that occur in warm and cool habitats have a long total reproductive season (Fig. 2; Beljaev, 1996; Nakajima, 1998), we designated all the species as either early winter moths or late winter moths according to their characteristics in cool habitats. Thus, the early winter moths comprised *Inurois fletcheri*, *Inurois nikkoensis*, *Inurois asahinai*, *Inurois kobayashii*, *Inurois kyushuensis*, *Inurois minutulus*, *Inurois fumosa*, *Inurois brunneus*, *Inurois* sp., and the early population of *I. punctigera*, whereas the late winter moths were *Inurois tenuis*, *Inurois membranaria*, *Inurois viidaleppi*, and the later population of *I. punctigera*.

We reconstructed the ancestral character states for reproductive timing using the program BayesMultistate in BayesTraits version 2 (Pagel and Meade, 2013), which can consider phylogenetic uncertainty in the analysis (Pagel et al., 2004). In this analysis, we used 8000 species trees obtained from an additional *BEAST analysis with the three nuclear genes, but we excluded specimens of *I. punctigera* from warm habitats because we could not assign characters to them. The parameter settings and the Bayesian MCMC analysis method were the same as those used for the JML analysis.

To estimate the ancestral characters with BayesMultistate, 110 million MCMC iterations were conducted after a burn-in of 10 million iterations with the reversible-jump hyperprior (exponential between 0 and 30) option. The results of each MCMC were sampled every 10,000 iterations. We tested the plausibility of the ancestral character state (early or late) using a Bayesian model comparison approach with the "fossil option" in BayesMultistate, where the test statistic that employed the Bayes factor was given as 2(log [harmonic mean(early winter model)] – log[harmonic mean(late winter model)]), and absolute values of the statistic greater than 2, 5, and 10 were considered to provide "positive," "strong," and "very strong" evidence, respectively, for a better model (for early or late winter), according to the BayesTraits manual.

3. Results

3.1. Phylogeny

The nuclear gene tree constructed by ML method (Fig. 3a) was almost identical to the Bayesian consensus tree (not illustrated here). The tree based on the combined nuclear + morphological data was also identical to the nuclear gene tree (Fig. S1). In the nuclear tree, each of *I. punctigera*, *I. brunneus*, and *Inurois* sp. was monophyletic with high node support, and they formed a monophyletic clade. *Inurois membranaria* was also monophyletic. On the other hand, the monophyly of species was not recovered for the monophyletic group of *I. asahinai*, *I. kobayashii*, *I. kyushuensis*, and *I. minutulus* (referred to as the *asahinai* group) or for another monophyletic group that comprised *I. fletcheri* and *I. nikkoensis* (referred to as the *fletcheri* group). The sister relationship between the *asahinai* and *fletcheri* groups and *I. membranaria* was suggested by both ML tree and the Bayesian consensus tree with relatively weak supports (Fig. 3). However, this tree differed significantly from an alternative ML tree in which *I. membranaria* is sister to the clade including all the species except *I. tenuis*, *I. viidaleppi*, and *I. fumosa* (AU test; difference in log likelihood = 2.7, $P = 0.036$). The lineage that comprised the *asahinai* and *fletcheri* groups and *I. membranaria* was a sister to the lineage that comprised *I. punctigera*, *I. brunneus*, and *I.* sp. (Fig. 3). *Inurois tenuis*, *I. fumosa*, and *I. viidaleppi* were each monophyletic, after ignoring three exceptional specimens (tenuis_F1180, fumosa_F359, and viidaleppi_F1428). *Inurois fumosa* and *I. viidaleppi* formed a well-supported clade with *I. tenuis* (Fig. 3).

The COI genealogies constructed using the ML (Fig. 3b) and Bayesian methods (not illustrated) were almost identical to each other. In particular, *I. fumosa* was separated into two distant groups, one of which was related to *I. membranaria*, *I. brunneus*, and *I. viidaleppi* (group A), whereas the other was closely related to *I. tenuis* (group B; Fig. 3b). In group A of *I. fumosa*, one haplotype was shared with *I. tenuis*. Species were not monophyletic within the *asahinai* and *fretcheri* groups, as found in the nuclear gene tree. Thus, the phylogeny based on the mitochondrial gene differed considerably from that based on the nuclear genes.

3.2. Mitochondrial introgression

We tested the hypothesis of mitochondrial introgression with three values of the relative substitution rate of COI gene and obtained almost identical results from the analyses using the values of 3.79 and 5.94 (Tables S3 and S4; summarized in Table 2). The results show that at least one COI haplotype was significantly less distant than the species distance simulated by coalescent theory (i.e. the null distribution of the genetic distance between species; Table 2) in three pairs (*I. membranaria–I. fumosa*, *I. membranaria–I. viidaleppi*, and *I. membranaria–I. tenuis*). In the pair of *I. membranaria* and *I. fumosa* group A haplotypes, all the COI distances between two species were significantly smaller than the simulated species distances (Tables S3 and S4). Similarly, the haplotype distances were significantly smaller than the species distance between *I. membranaria* and *I. viidaleppi*, except for some haplotype distances with slightly higher P values (0.0021–0.0037), but they were not significant after controlling for FDR at $\alpha = 0.05$. However, the significant sign for introgression between *I. membranaria* and *I. tenuis* was attributable to a single *I. tenuis* haplotype (F1194_tenuis). This haplotype was shared with individuals of *I. fumosa* in group A (e.g., the COI sequences of fumosa_F224 and fumosa_F561 were identical to that of tenuis_F1194; Fig. 3b and Table S4).

The result from analysis using the value of 8.45 suggested a few additional introgression events: introgressions among *I. membranaria*, *I. viidaleppi*, *I. brunneus* and *I. fumosa*, and those between *I. viidaleppi* and *I. nikkoensis* (Table S5). Because *I. viidaleppi* does not occur in Japan and *I. nikkoensis* occurs only in Japan, these species would have never contacted with each other, suggesting that the introgression could be a result of overestimation. Therefore,

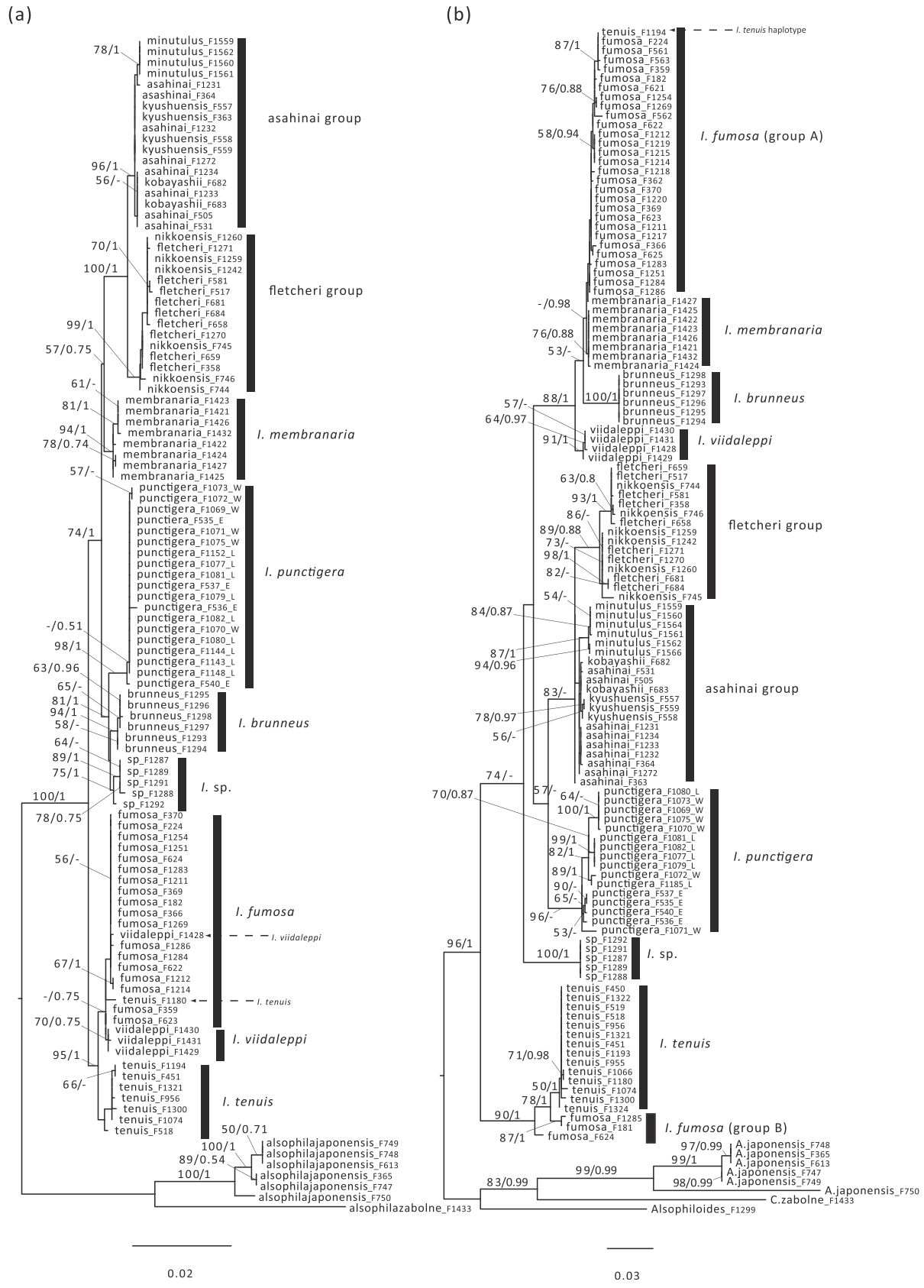

Fig. 3. Maximum-likelihood (ML) tree based on the nuclear gene sequence data (a) and based on the cytochrome oxidase subunit I (COI) sequence data (b). The Bayesian tree is not shown, but it was almost identical to the ML tree. The Bayesian tree based on COI data was also almost identical to the ML tree based on COI data. The numbers near the branches are the node support values: bootstrap probability (BP)/Bayesian posterior probability (PP). Note that the node support values of >50% are shown. *Inurois punctigera* from early and late winter populations are indicated by "E" and "L", respectively, and *I. punctigera* from warm habitats where seasonal segregation of flight period is absent by "W".

Table 2
Summary of the test results for mitochondrial gene introgression between *Inurois* species. Only the minimum sequence distance of cytochrome oxidase subunit I haplotypes between the pair of species is given (see Table S4 for details).

Species pair	Minimal distance	Probability[1]
I. membranaria; *I. fumosa*	0.0011	0.00025
I. membranaria; *I. viidaleppi*	0.0111	0.00075
I. membranaria; *I. tenuis*	0.0067	0.00025

[1] All values are significant after controlling for FDR at $\alpha = 0.05$.

we summarize the results from the analysis with the mean substitution rate here (Table 2).

3.3. Ancestral character states

The consensus species tree constructed by *BEAST (Fig. 4) was consistent with the tree based on the nuclear genes. Table 3 shows the estimated probabilities of the ancestral character states (early or late winter; see also Fig. 4). The probabilities of each character state exceeded 75% for nodes B, D, E, G, and I. For these nodes, the character state with a higher probability was likely to be the ancestral state based on the Bayes factor. In addition to these nodes, the late winter state exceeded 0.5 at node C (common ancestor of *I. viidaleppi* and *I. tenuis*), as did the early winter state at node F (common ancestor of early and late winter *I. punctigera*), both of which were supported by the Bayes factors. Only two nodes produced equivocal results based on the Bayes factor: node A (common ancestor of genus *Inurois*) and node H (common ancestor of *I. membranaria*, and the *fletcheri* and *asahinai* groups), although the probabilities of the early winter state were higher at both nodes.

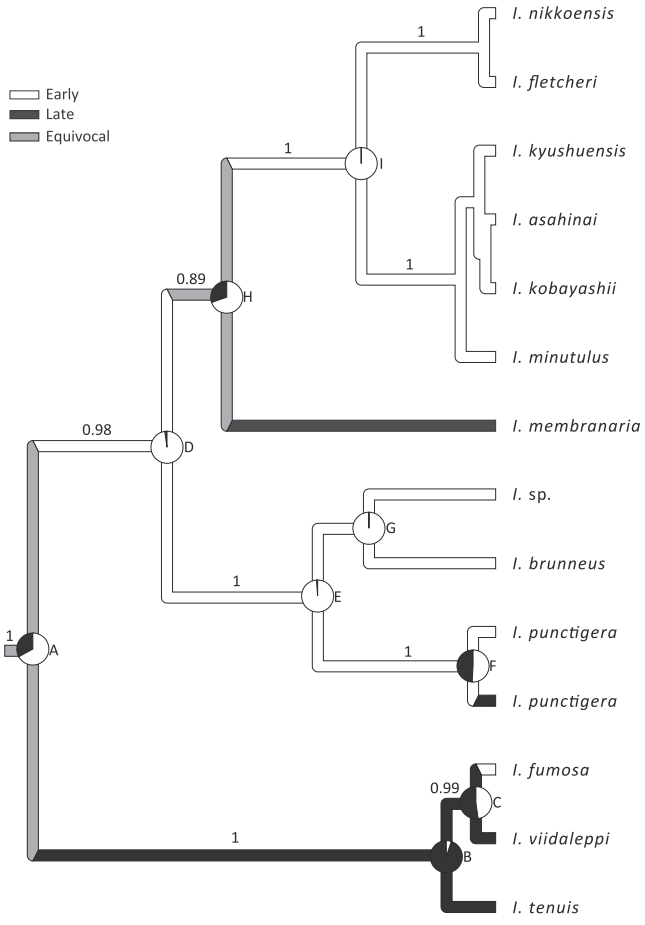

Fig. 4. Inferred species tree with a summary of inferred character evolution. For the ancestral character states at nodes A–I, the inferred probabilities given in Table 2 are represented using pie charts on the nodes. The colors of the branches indicate the estimated character states: white, early winter moth; black, late winter moth; gray, equivocal. Equivocal character states indicate that the estimated ancestral characters were not significant in terms of the Bayes factor ($-2 <$ Bayes factor < 2). The tree topology was the consensus species tree based on the nuclear sequence data, excluding the population of *I. punctigera* in a warm habitat (see Section 2) and the posterior probabilities (>0.80) for each node.

Table 3
Probabilities of the character states at ancestral nodes A–I in Fig. 3 and the test statistics used to determine whether early or late winter reproduction was likely to be the ancestral state.

Node[1]	Probability		
	Early	Late	Test statistic[2]
A	0.67	0.37	1.7806
B	0.05	0.95	**4.2611**
C	0.48	0.52	**3.8025**
D	0.98	0.02	**−7.3510**
E	0.99	0.01	**−8.8125**
F	0.51	0.49	**−6.4675**
G	1.00	0.00	**−15.388**
H	0.70	0.30	−1.9793
I	1.00	0.00	**−12.3060**

[1] Nodes correspond to those in Fig. 4.
[2] Test statistic: 2{log[harmonic mean(likelihood of early winter model)] − log [harmonic mean(likelihood of late winter model)]}. Absolute values of the test statistic greater than 2, 5 and 10 provided 'positive', 'strong' and 'very strong' evidence, respectively, for early winter (when >0) or late winter (when <0) ancestors. Values in bold suggest positive statistical evidences.

4. Discussion

Our phylogenetic analysis demonstrated that the temporal segregation of reproductive activities has occurred repeatedly within the *Inurois* lineage. Reproductive season shifts occurred in the common ancestor of the genus *Inurois* (node A; Fig. 4), as well as during the divergence of *I. viidaleppi* and *I. fumosa* (node C), the divergence of early and late winter *I. punctigera* (node F), and the divergence of *I. membranaria* and the *fletcheri* and *asahinai* groups (node H). Thus, assuming that the early and late winter *I. punctigera* are different species, evolutionary divergence of the reproductive season might have occurred four times during 13 speciation events in *Inurois*. However, the order of phylogenetic divergence among species should affect the interpretation of the number of reproductive season shifts, and our phylogenetic analysis showed weak support for the position of *I. membranaria*. On the other hand, the AU test significantly supported the presented nuclear gene tree (Fig. 3a) rather than alternative phylogenetic hypothesis. Thus, about 30% of the speciation events might be associated with reproductive season shifts.

Our results suggest how allochronic isolation may act among sister species with opposite reproductive seasons in the same region. First, an early and a late winter species, *I. viidaleppi* and *I. fumosa*, are probably sister species (Figs. 3 and 4), and their species ranges overlap with each other (Fig. 2). Sister species with obvious differences in reproductive timing should indicate the importance of temporal isolation in speciation (Coyne and Orr, 2004). The reproductive timing shift from late winter to early winter would have isolated the ancestral *I. fumosa* population from *I. viidaleppi*, although there is the potential that their initial divergence have been facilitated by a geographic barrier. Two further studies are needed to evaluate the importance of allochronic isolation between *I. viidaleppi* and *I. fumosa*: a phylogeographic study based on intensive collections from the entire ranges of the two species,

and tests of mating preferences and hybrid fertility. Second, in another divergence event associated with a reproductive season shift between *I. membranaria* (late winter species) and an ancestor of the *fletcheri*/*asahinai* groups (early winter species; Figs. 3 and 4), geographic isolation appears to have been more crucial than allochronic isolation because *I. membranaria* occurs only in Russia and the Korean peninsula, whereas all the species in the *fletcheri*/*asahinai* groups occur only in Japan. Thus, our study suggests that allochronic sister species may occur both in sympatry and allopatry. However, our phylogenetic analyses have not provided fully resolved, robust topologies for the species relationships, especially for the position of *I. membranaria* (Figs. 3 and 4). To reveal the geographic mode of each speciation event, a more robust species tree is needed.

The population divergence caused by reproductive timing segregation is considered to be fragile, and it operates only weakly in general (Mayr, 1963; Devaux and Lande, 2008; Matsumoto et al., 2013). Indeed, in our previous study, we demonstrated that the previously diverged early and late winter populations of *I. punctigera* merged in warm habitats (Yamamoto and Sota, 2009, 2012). In the present study, we found a possible case of introgressive hybridization between early winter (*I. fumosa*) and late winter (*I. membranaria*) species, as well as one between late winter species (*I. viidaleppi* and *I. membranaria*), thereby suggesting introgression between species with opposite reproductive seasons. However, these cases may be explained together without assuming that independent mitochondrial introgression occurred between *I. fumosa*/*I. viidaleppi* and *I. membranaria*. First, given that *I. fumosa* and *I. viidaleppi* are sister species (Figs. 3 and 4), and that no identical COI haplotypes were shared among *I. fumosa*, *I. viidaleppi*, and *I. membranaria*, introgressive hybridization may have occurred between *I. membranaria* and the common ancestor of *I. fumosa* and *I. viidaleppi*, which was estimated to be a late winter species (Table 3; Fig. 4). The age of this hybridization event might be sufficiently ancient to have accumulated some mutations. This hypothesis does not include hybridization between species with opposite reproductive seasons. Alternatively, *I. membranaria* may have hybridized with either *I. fumosa* or *I. viidaleppi* and "apparent" introgression might be detected by the JML analysis because *I. fumosa* and *I. viidaleppi* are very close to each other (Fig. 3a). Considering *I. membranaria* is closer to *I. fumosa* than *I. viddapleppi* in the mitochondrial gene tree, *I. membranaria* might hybridize with *I. fumosa*. If so, the possibility remains that introgression has occurred between species with opposite reproductive seasons. In addition, we detected one identical haplotype shared between early and late winter species, *I. fumosa* and *I. tenuis* (Fig. 3b). However, this case was not significantly attributed to introgressive hybridization in the JML test using relative substitution rate of 5.94, and it was barely significant even using the fastest value of 8.45. Thus, although we cannot clearly conclude about the occurrence of mitochondrial introgression in *Inurois* species, hybridization between species with opposite reproductive seasons is apparently not common, and reproductive season segregation may have contributed to reproductive isolation between early and late winter species of *Inurois*.

In conclusion, we showed that four of 13 speciation events (including one polymorphic case) during the divergence of *Inurois* were associated with disjunction of the reproductive seasons between early and late winter. Reproductive season segregation may also have contributed to the diversification of other winter moth groups. For example, it could have been important in the divergence of *Alsophila*, which is related to *Inurois*, and *Larerannis*, which is phylogenetically independent from *Inurois* and *Alsophila*. Each of these genera also include both early and late winter species (Beljaev, 1996; Nakajima, 1998). Thus, temporal segregation of reproductive activities caused by harsh winter conditions may have promoted the species diversification of moths that reproduce in winter.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.08.016.

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