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Reassessment of the status of *Lymantria albescens* and *Lymantria postalba* (Lepidoptera: Erebidae: Lymantriinae) as distinct 'Asian gypsy moth' species, using both mitochondrial and nuclear sequence data

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Abstract. For regulatory purposes, the name 'Asian gypsy moth' refers to a group of closely related Asian *Lymantria* species and subspecies whose female moths display flight capability, a trait believed to confer enhanced invasiveness relative to the European gypsy moth, *Lymantria dispar dispar*, whose females are flightless. *Lymantria albescens* and *Lymantria postalba* are Asian gypsy moths occurring in the southern Ryukyu Islands and in the northern Ryukyu and adjacent Kyushu and Shikoku Islands of Japan, respectively. Although once considered subspecies of *L. dispar*, their status as distinct species, relative to the latter, is now well established. While *postalba* was subsequently considered a subspecies of *L. albescens*, largely on the basis of differences in forewing ground colour in males, both taxa were later given distinct species status by Pogue & Schaefer (2007) following their revision of the genus *Lymantria*. Here, we re-examined the validity of this revised status through the sequencing of a large portion of the mitochondrial genome (*c*. 60%) and multiple nuclear marker genes [elongation factor 1-alpha (Ef-1α), wingless (Wgl), internal transcribed spacer 2 (ITS-2), ribosomal protein S5 (RpS5)] in representative specimens of both taxa and other *Lymantria* species, including *L. monacha*, *L. xylina*, *L. mathura* and members of the *L. dispar* + *L. umbrosa* clade. A comparison of the number of substitutions in these genomic regions among the taxa we considered showed lower or equivalent variation between *L. albescens* and *L. postalba* compared with subspecies of *L. dispar*, for mitochondrial and nuclear sequences, respectively. This finding was reflected in the maximum likelihood trees generated independently for mitochondrial and nuclear data, where *L. albescens* and *L. postalba* formed, in both analyses, a short-branch sister clade basal to the *L. dispar* + *L. umbrosa* clade. We further sequenced three markers [cytochrome *c* oxydase 1 (COI), EF-1α, Wgl] in multiple *L. albescens*–*L. postalba* specimens collected along a south-to-north transect across the Ryukyu Arc and observed no clear distinction

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among the sampled specimens as a function of taxonomic designation. We conclude that *L. albescens* and *L. postalba* form a single species, with *postalba* representing a darker-winged morph along an apparent south-to-north wing colour cline. Accordingly, *L. postalba* is relegated to synonymy under *L. albescens* (**syn.n.**).

Introduction

Preventing accidental introductions of 'Asian gypsy moths' (AGMs) into North America is a high-priority goal for national plant protection organizations such as the Canadian Food Inspection Agency (CFIA) in Canada, and the Animal and Plant Health Inspection Services (APHIS) in the United States. Given the flight capability of their females (Keena *et al.*, 2008) and the very broad host range of their larvae (> 500 tree species; Baranchikov, 1989), AGMs represent a significantly greater invasion threat than their North American counterpart, the European gypsy moth (EGM). The latter was accidentally introduced into Massachusetts from Europe in 1869 and is now established over most of north-eastern North America (Liebhold *et al.*, 1989; Keena *et al.*, 2008). There, EGM is responsible for recurrent outbreaks and severe economic losses (Bradshaw *et al.*, 2016).

European and Asian gypsy moths form a group of closely related species and subspecies that belong to the genus *Lymantria* (family Erebidae). Whereas the name EGM refers to a single taxon, *L. dispar dispar* (L.), the term AGM currently designates a collection of five taxa, *L. dispar asiatica* Vnukovskij, *L. dispar japonica* Motschulsky, *L. umbrosa* Butler, *L. albescens* Inoue, and *L. postalba* Hori and Umeno; this designation is based on the observation that female moths of all five Asian taxa are flight-capable, unlike those of *L. dispar dispar* (Pogue & Schaefer, 2007). For regulatory purposes, both the CFIA (CFIA, 2018) and APHIS (USDA, 2016) have adopted Pogue & Schaefer's (2007) criteria for designating these lymantriines as 'AGM'.

It is worth noting that *L. umbrosa*, *L. albescens* and *L. postalba* were once considered subspecies of *L. dispar* (*L. dispar umbrosa* Matsumura 1933; *L. dispar albescens* Hori and Umeno 1930; *L. dispar postalba* Inoue 1956; see Pogue & Schaefer, 2007), while revisions of the genus *Lymantria* by Schintlmeister (2004) led to *albescens* being elevated to the species level (*L. albescens albescens* Schintlmeister 2004) and *postalba* being designated a subspecies of *L. albescens* (*L. albescens postalba* Schintlmeister 2004). The distinct species designations for these three taxa resulted from taxonomic revisions conducted by Pogue & Schaefer (2007), which have since more or less prevailed.

We recently developed a qPCR TaqMan assay aimed at the rapid molecular identification of all AGM taxa, plus EGM and five other invasive lymantriines (Stewart *et al.*, 2016). This type of assay relies on the identification of taxon-specific molecular signatures, which are then targeted by qPCR primers and TaqMan probes; for our purposes, these molecular signatures were provided by single nucleotide polymorphisms (SNPs) found in the mitochondrial (mt) *cytochrome c oxydase 1* (COI) gene. Such unique signatures could be found for all targeted taxa, except for *L. albescens* and *L. postalba*, which we could easily distinguish from the other targeted *Lymantria* species and subspecies, but not from one another; as a consequence, these two taxa were treated as a single taxon in our assay (Stewart *et al.*, 2016; see also Stewart *et al.*, 2019).

This observation led us to call into question the species status attributed to *L. albescens* and *L. postalba* by Pogue & Schaefer (2007), who described the two taxa as being distinguishable on the basis of differences in forewing length and forewing ground colour in males. In addition, they described the two taxa as having rather distinct geographic ranges, with *L. albescens* occurring in the southern Ryukyu Islands of Japan, including Okinawa, and *L. postalba* occurring on the more northern Ryukyu Islands, plus Kyushu, Shikoku, Tsushima and the Kii Peninsula (see Fig. 1). Two subsequent studies concurred with Pogue & Schaefer's revised status for *albescens* and *postalba*: while the conclusions of one were based on morphological criteria alone (Arimoto & Iwaizumi, 2014a), those of the other study were based on the presence of a single SNP within the *cytochrome c oxydase 2* (COII) gene, sequenced from only one specimen each of *albescens* and *postalba* (Arimoto & Iwaizumi, 2014b). However, the same authors subsequently conducted a more thorough molecular assessment, based on partial COI and COII sequences, and concluded that the two taxa could not be distinguished from one another on the basis of these mt genomic data (Arimoto & Iwaizumi, 2018).

In an effort to bring closure to the taxonomic conundrum described above, we undertook the sequencing of a large portion of the mt genome [ten protein-coding genes (PCGs) and 12 transfer RNA (tRNA) genes] in representative specimens of *L. albescens* and *L. postalba*, plus those of *L. monacha* (L.), *L. xylina* Swinhoe and *L. mathura* Moore, for comparative purposes. Our analyses also included the corresponding mt sequences obtained earlier for *L. umbrosa* and geographic variants of *L. dispar* (Djoumad *et al.*, 2017). Finally, we also sequenced four nuclear marker genes from the same specimens used for mt sequence analysis. Our results led us to the conclusion that *albescens* and *postalba* are, at best, two morphs of a single species.

Materials and methods

Insect specimens

Sources of *Lymantria* specimens used in this study are provided in Tables 1 and 2. Moths were obtained either as specimens preserved at −80°C, or as archival, dry adults. Species/subspecies identification was verified by CFIA taxonomists on the basis of morphological characters (Pogue &

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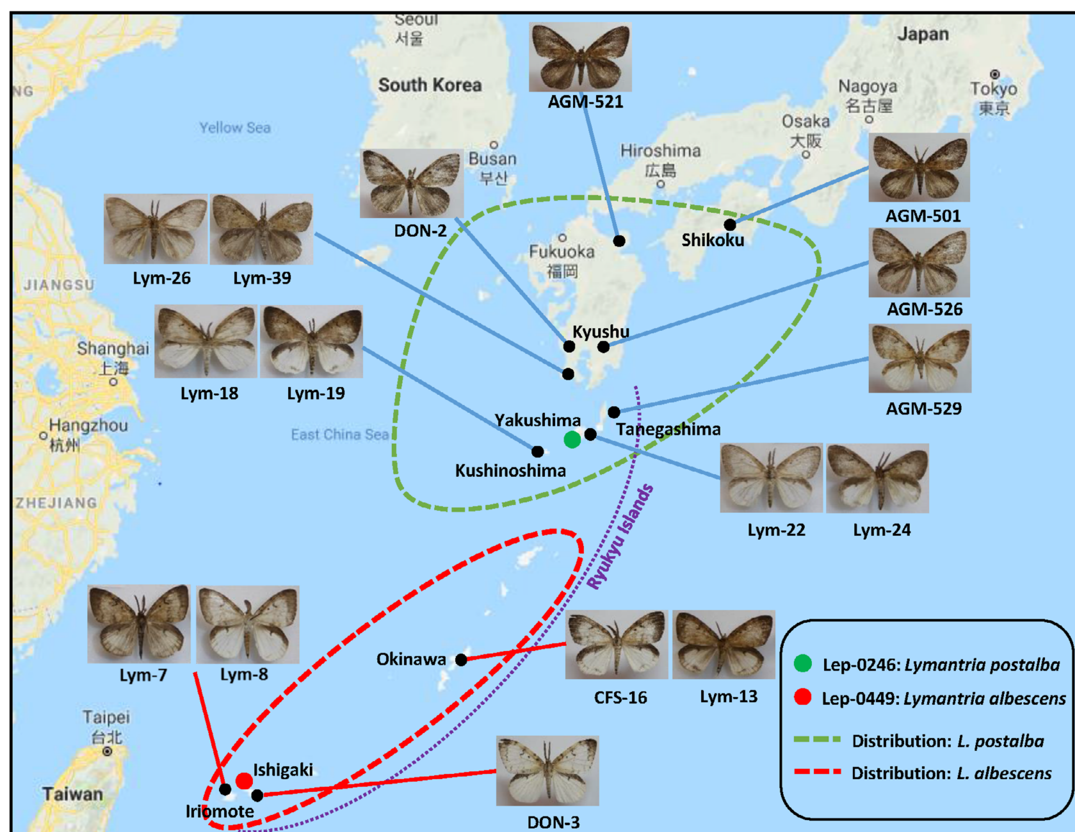

Fig. 1. Geographical distribution of *Lymantria albescens* and *Lymantria postalba* specimens used for genomic analyses. Green and red dots represent location where the two main specimens of *L. postalba* (Lep-0246) and *L. albescens* (Lep-0449), used for partial mitochondrial genome sequencing, were collected. Black dots indicate sampling locations of the other specimens used in phylogenetic analyses (background map generated using Google Maps). [Colour figure can be viewed at wileyonlinelibrary.com].

Table 1. *Lymantria* specimens used for mitogenome sequencing, along with GenBank accession numbers

Species/subspecies	Code	Origin	Region	Supplier	Accession number
L. albescens [a]	Lep-0449	Japan	Ichigaki Island	C. Hideyuki	MH388823
L. postalba [a]	Lep-0246	Japan	Yakushima Island	K. Ueda	MH388826
L. xylina	–	Taiwan	Yilan	S. Wu	MH388827
L. mathura	–	Russian Far East	Nakhodka	H. Nadel	MH388824
L. monacha	–	Czech Republic	–	M. Keena	MH388825
L. dispar dispar [b]	Ldd_NJ	USA	New Jersey	M. Keena	KY798442
L. dispar dispar [b]	Ldd_KG	Greece	Kavála, Macedonia	M. Keena	KY923062
L. dispar asiatica [b]	Ldd_TJ	China	Tianjin	H. Nadel	KY923067
L. dispar japonica [b]	Ldj_ID	Japan	Iwate district, Honshu	H. Nadel	KY923060
L. umbrosa [b]	Lu_JH	Japan	Hokkaido	C. Hideyuki	KY923066
L. dispar? [b]	L?_IR	Iran	Mazandaran, Noor	H. Rajaei	KY923068

[a]Voucher specimens available at the Canadian National Collection of Insects, Arachnids, and Nematodes (CNC), Ottawa, Canada.
[b]Reported earlier (Djoumad *et al.*, 2017).

Schaefer, 2007), and subsequently confirmed by us on the basis of the COI barcoding sequence, except for *L. albescens* and *L. postalba*, which could not be distinguished using this barcoding approach alone (Stewart *et al.*, 2016). Because morphological traits did not always provide unequivocal resolution between these two taxa, geographic origin was used as a confirmatory criterion (see Introduction for details).

Total DNA extraction

Tissue obtained from frozen and archival specimens (one or two legs in most cases) was ground in liquid nitrogen before DNA extraction. Total genomic DNA (nuclear and mt) was extracted using the Qiagen Blood & Cell Culture DNA midi kit or the Qiagen DNeasy Blood & Tissue Mini Kit (Qiagen,

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Table 2. *Lymantria postalba* and *Lymantria albescens* specimens from which the elongation factor 1-alpha, wingless and cytochrome *c* oxydase 1 markers were sequenced and used in phylogenetic analysis[a]

Species	Code	Origin	Region	Supplier
L. postalba	AGM-501	Japan	Hiranabe, Shikoku	M. Kimura
	AGM-521	Japan	Karisyuku, Kyushu	M. Kimura
	AGM-526	Japan	Uchikidani, Kyushu	M. Kimura
	AGM-529	Japan	Tanegashima	M. Kimura
	DON-2	Japan	Kagoshima, Kyushu	T. Fukuda
	Lym-18	Japan	Kuchinoshima	T. Moriyama
	Lym-19	Japan	Kuchinoshima	T. Moriyama
	Lym-22	Japan	Yakushima	M. Kimura
	Lym-24	Japan	Yakushima	M. Kimura
	Lym-26	Japan	Kyushu	T. Fukuda
	Lym-39	Japan	Kyushu	M. Kimura
	Lep-0246	Japan	Yakushima	K. Ueda
L. albescens	CFS-16	Japan	Nekumachijidake, Okinawa	M. Kimura
	DON-3	Japan	Nosoko, Ishigaki	M. kimura
	Lym-7	Japan	Taketomi, Iriomote	M. Kimura
	Lym-8	Japan	Taketomi, Iriomote	M. Kimura
	Lym-13	Japan	Nekumachijidake, Okinawa	M. Kimura
	Lep-0449	Japan	Ishigaki	C. Hideyuku

[a]Voucher specimens available at the Canadian National Collection of Insects, Arachnids, and Nematodes (CNC), Ottawa, Canada.

Hilden, Germany), following the manufacturer's instructions, with minor modifications (see Djoumad *et al.*, 2017). After spectrophotometric quantification (NanoDrop ND-1000; Thermo Fischer Scientific Inc., Waltham, MA, U.S.A.), purified DNA was used for PCR amplification and sequencing, either directly or diluted.

PCR amplification and sequencing of partial mitochondrial genomes

Initial mitogenome analyses were based on sequences obtained from one adult male drawn from each of 11 *Lymantria* taxa/populations (Table 1; see also Fig. 1 for the geographical sources of the *L. albescens* and *L. postalba* specimens). While sequences for *L. umbrosa* and *L. dispar* subspecies were gleaned from an earlier study (Djoumad *et al.*, 2017), sequences for *L. albescens*, *L. postalba*, *L. xylina*, *L. monacha* and *L. mathura* were generated here from one specimen per species. With the exception of *L. mathura*, sequencing of these mitogenomes was limited to two regions containing the majority of PCGs. To this end, we employed either newly designed primers (Table S1) or primers designed in the context of an earlier study (Djoumad *et al.*, 2017) to amplify overlapping fragments of the two targeted regions, using 0.2–2 ng of purified DNA as template. Sequencing of amplicons was carried out at the Genome Sequencing and Genotyping Platform of the CHUL, Quebec City (Canada). With respect to *L. mathura*, a draft mitogenome sequence was first assembled from PacBio sequences generated in the context of an independent nuclear genome sequencing project (M. Cusson, unpublished data), using the Celera assembler (Myers *et al.*, 2000). This sequence was then subjected to quality control through targeted amplifications and complementary Sanger sequencing, as previously described (Djoumad *et al.*, 2017).

PCR amplification and sequencing of nuclear markers

In addition to mt genome sequencing, four nuclear markers, developed and used in previous studies (e.g. Pfeifer *et al.*, 1995; Wahlberg & Wheat, 2008; Wang *et al.*, 2015), were selected for phylogenetic analyses. These genomic regions were amplified and sequenced (Table S2) for the 11 species and subspecies listed in Table 1; these regions correspond to: (i) internal transcribed spacer 2 (ITS-2, *c.* 632 bp); (ii) nuclear elongation factor 1α (EF-1α, *c.* 541 bp), ribosomal protein S5 (RpS5, *c.* 617 bp) and wingless (Wgl, *c.* 450 bp). For PCR conditions, see references provided in Table S2. Sequencing of amplicons was conducted as described earlier for mitogenomes.

Mitochondrial genome annotation

Assembly and annotation of the partial mt genomes were carried out using GENEIOUS v.9.1.2 (http://www.geneious.com; Kearse *et al.*, 2012) and the NCBI Blast program for PCG predictions. For tRNA genes, tRNAscan-SE 1.21 (Lowe & Eddy, 1997; Schattner *et al.*, 2005) was used, with the search mode and the cove cutoff score set as default, mitochondria/chloroplast set as the search source, and invertebrate mitogenomes selected as the genetic code for tRNA isotype predictions. Finally, assembly of the two long mt DNA fragments generated for *L. postalba*, *L. albescens*, *L. xylina* and *L. monacha* was carried out using the mitogenome of *L. dispar dispar* (Djoumad *et al.*, 2017) as template.

Targeted sequencing of additional L. albescens *and* L. postalba *specimens*

Given that the described mt and nuclear sequences were generated for only one exemplar per taxon, we selected an

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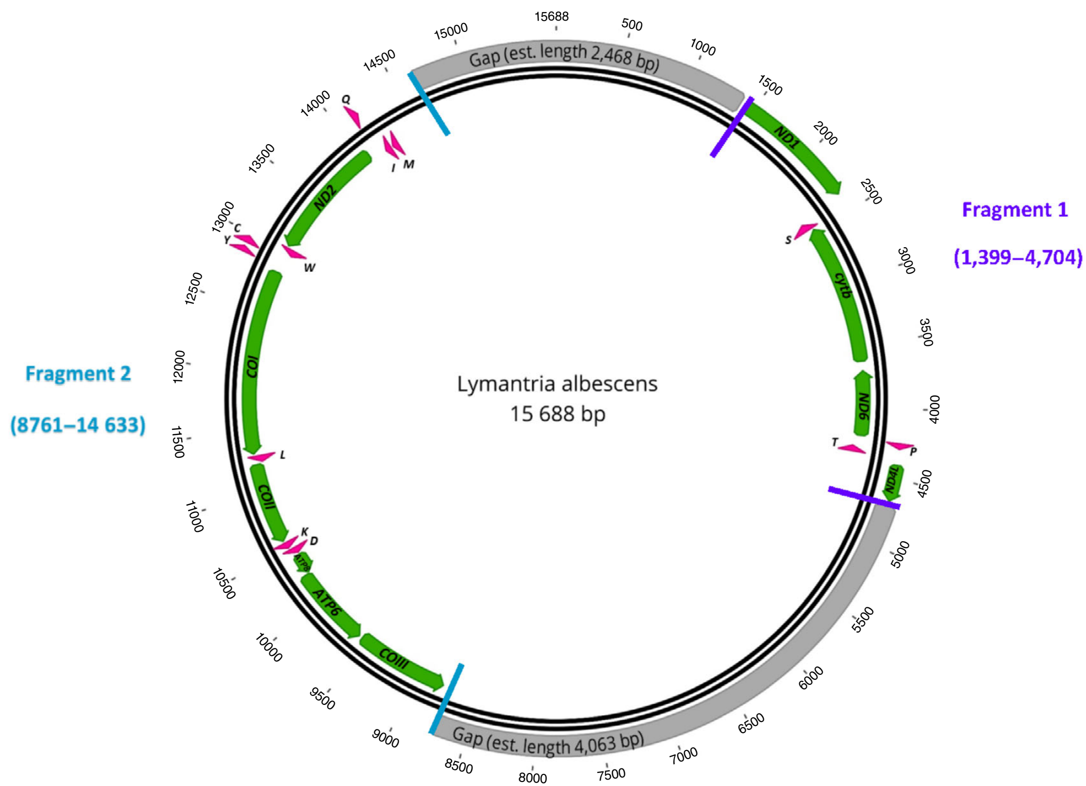

Fig. 2. Circular map of partial mitochondrial genome of *Lymantria albescens*. CO, cytochrome oxidase subunit; cyt b, cytochrome b; ND, NADH dehydrogenase. The transfer RNA genes are indicated using the single-letter IUPAC-IUB abbreviation, corresponding to their amino acid. Grey boxes, nonamplified mitochondrial genome regions; blue bars delimit the two amplified fragments. [Colour figure can be viewed at wileyonlinelibrary.com].

additional six specimens of *L. albescens* and 12 specimens of *L. postalba*, collected at locations spread across their geographic range (Fig. 1; Table 2), for further marker amplification and sequencing. For this purpose, we focused on the COI mt barcoding region and two nuclear markers, Ef1α and Wgl. These three marker sequences were concatenated into a single 1041 bp fragment, which was then used for phylogenetic analysis.

Phylogenetic analyses

Phylogenetic relationships among the 11 populations considered here (Table 1) were inferred using two types of data: (i) the ten PCGs plus the 12 tRNA genes obtained through partial mitogenome sequencing; and (ii) the four nuclear markers. Mitochondrial and nuclear gene regions were analysed using model-based phylogenetic approaches (e.g. maximum likelihood, ML). The data matrices were first analysed in various combinations to explore their phylogenetic signal. For example, single genes were analysed on their own, nuclear genes were combined, third codon positions were removed, data were partitioned into mtDNA and nuclear DNA (nDNa) and, finally, the data were partitioned by gene region (22 mtDNA+ four nDNA partitions). The effects of modifying taxon composition (e.g. excluding *L. mathura* and *L. monacha* as outgroups) and varying gene combinations were compared against analyses of the full, combined and partitioned dataset. Based on these data explorations, we decided to include all genes and third codon positions and to partition the data by genes in ML analyses; inclusion of all genes and varying partitioning strategies had no major impact on bootstrap support values, either among closely related species or at basal nodes. The best-fit models according to Bayesian information criterion scores, as assessed using MODELFINDER (Kalyaanamoorthy *et al.*, 2017), were chosen as GTR + F + I for the mtDNA dataset and TNe + 4 for the nDNA dataset. The most appropriate model of sequence evolution for the combined (concatenated) dataset was GTR + F + G4. The ML and ML bootstrap analyses were conducted using the IQ-TREE web server (Trifinopoulos *et al.*, 2016). The same approach was employed for phylogenetic analysis of the additional *L. albescens* and *L. postalba* specimens.

Results

Mitochondrial genome sequencing and annotation

The partial mitogenomes of *L. albescens* and *L. postalba*, plus those of *L. xylina* and *L. monacha*, were obtained through the assembly and annotation of the two fragments depicted in Fig. 2, while corresponding fragments were gleaned from the full mitogenome of *L. mathura* (see Materials and Methods for

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details). The larger of these fragments (fragment 1–5864 bp) contained six PCGs, including the three *cytochrome c oxidase* genes (*COI*, *COII* and *COIII*), two *ATP synthase* genes (*ATP6* and *ATP8*) and one *NADH dehydrogenase* gene (*ND2*). In comparison, the smaller fragment (fragment 2–3291 bp) harboured the *ND1*, *ND6*, *ND4L* genes and the *cytochrome b oxidase* (*cytb*) gene. In addition, fragments 1 and 2 featured nine and three tRNA genes, respectively. Altogether, the two fragments represented approximately 60% of the complete mitogenomes, amounting to 10 of the 13 known mt PCGs and 12 of the 22 tRNAs (Fig. 2), and displayed a conserved gene arrangement relative to other lepidopteran mitogenomes (Cameron & Whiting, 2008; Sun *et al.*, 2016; Djoumad *et al.*, 2017).

Mitogenomes of L. albescens *and* L. postalba *versus those of other* Lymantria *species*

In order to assess the degree of divergence between the mitogenomes of *L. albescens* and *L. postalba*, we first examined nucleotide variation between these two taxa and compared it with that observed among other *Lymantria* species and subspecies, namely *L. umbrosa*, *L. monacha*, *L. xylina* and *L. mathura*, plus exemplars of the recognized subspecies of *L. dispar*, including a recently described genotypic entity from the Caucasus region (Djoumad *et al.*, 2017; Zahiri *et al.*, 2019) (see Table 1). To this end, we aligned the two assembled mitogenome fragments and produced a diagram where each nucleotide substitution relative to the *L. albescens* sequence is illustrated by a black vertical bar (Fig. 3). The near absence of nucleotide variation between the *L. albescens* and *L. postalba* mitogenome fragments is conspicuous on this diagram, attesting to the high genetic proximity of these two genomes. With only 15 SNPs separating *L. albescens* from *L. postalba* (within the genome fragments under consideration; Table 3; Fig. 3), the level of nucleotide substitution between these two taxa was lower than that observed between subspecies of *L. dispar*, namely between EGM (Ldd_NJ and Ldd_KG) and AGM (Lda_TJ and Ldj_ID), where we observed an average of 61 substitutions (Table 3). Even the North American and European exemplars of *L. d. dispar* showed a higher level of substitution (34 SNPs), relative to one another, compared with the *L. albescens*–*L. postalba* pair.

As expected, other *Lymantria* species known to be clearly distinct from one another (*L. umbrosa*, *L. xylina*, *L. monacha* and *L. mathura*) displayed high levels of substitutions relative to *L. albescens* and *L. postalba*, as well as to one another (Fig. 3; Table 3).

Nuclear markers

Inasmuch as nuclear and mt genomes are subjected to different evolutionary constraints, the occurrence of mitonuclear discordance is relatively common, especially in instances where hybridization and introgression have occurred (Toews & Brelsford, 2012). For this reason, our analyses also included the sequences of four nuclear markers frequently used in phylogenetic reconstructions. While RpS5 and Ef-1α exhibited limited polymorphism (except for the more distant outgroup species, *L. mathura* and *L. monacha*), ITS2 and Wgl displayed a higher proportion of informative substitutions and indels (ITS2 only; data not shown). Significantly, *albescens* and *postalba* were separated by only three SNPs over the four concatenated nuclear sequences, a value that is similar to the numbers of substitutions observed among *L. dispar* subspecies (Table 3). In comparison, there were *c*. 20 SNPs separating *L. albescens*/*L. postalba* from *L. dispar* or *L. umbrosa*, while *c*. 80, 210 and 330 SNPs separated them from the outgroup species *L. xylina*, *L. monacha* and *L. mathura*, respectively (Table 3).

Phylogenetic analyses based on partial mitogenomes and four nuclear markers

Maximum likelihood phylogenetic reconstructions computed from the partial mitogenomes (ten PCGs +12 tRNAs) and four nuclear markers (ITS-2, EF-1α, Wgl, RpS5) sequenced from 11 *Lymantria* exemplars, including *L. albescens* and *L. postalba*, are shown in Fig. 4. Both trees show *L. albescens* and *L. postalba* as forming a distinct, short-branch clade, immediately basal to the *L. dispar* + *L. umbrosa* clade. Whereas the partial mitogenomes successfully resolved members of the *L. dispar* + *L. umbrosa* clade (Fig. 4A), revealing a tree topology identical to that reported earlier for these taxa, based on full mitogenomes (Djoumad *et al.*, 2017), the four nuclear markers used here failed to recover this topology, providing resolution only for *L. umbrosa* (Fig. 4B). However, the three outgroups occupied similar positions at the base of each tree (Fig. 4). Not surprisingly, analysis of the concatenated mt and nuclear sequence data yielded a tree very similar to that presented in Fig. 4A (see Fig. S1).

Phylogenetic analysis using multiple exemplars of L. albescens *and* L. postalba

Given that a single exemplar each of *L. albescens* and *L. postalba* was used in the phylogenetic reconstruction presented above (Fig. 4), we conducted a separate phylogenetic analysis based on the mt COI barcode region, plus the nuclear Ef1α and Wgl markers, sequenced from a total of six *L. albescens* and 12 *L. postalba* specimens collected in their respective ranges (Fig. 1). The ML tree presented in Fig. 5 shows very clearly that presumptive *L. albescens* and *L. postalba* specimens cannot be resolved based on these three combined markers.

Discussion

Adults of some lepidopteran species have been observed to display important intraspecific variation in wing patterns. Perhaps the most striking example of this phenomenon is that provided by *Heliconius* butterflies, where species such as *H. erato* and *H. melpomene* have diversified into many different 'morphs',

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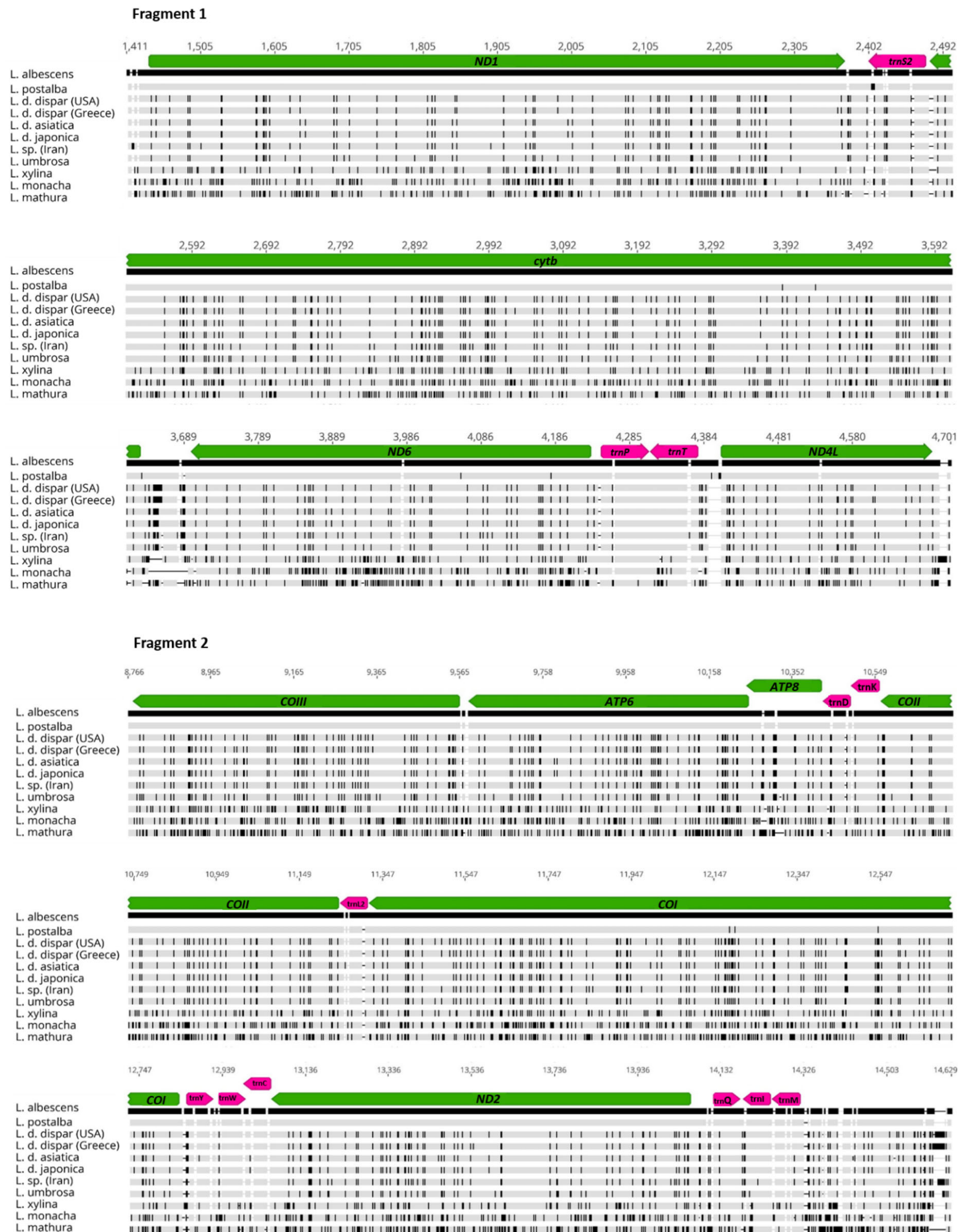

Fig. 3. Graphical representation of genetic diversity among the 11 partial mitochondrial genomes considered in the present study, following nucleotide sequence alignment of the two fragments we generated. Green boxes represent annotations corresponding to protein-coding genes. Pink arrows refer to the sequenced transfer RNAs; black vertical lines represent single nucleotide variations relative to the *Lymantria albescens* mitochondrial genome, shown here as reference (top horizontal black line). [Colour figure can be viewed at wileyonlinelibrary.com].

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Table 3. Numbers of pairwise nucleotide substitutions among 11 *Lymantria* taxa, as determined for two mitogenome fragments (upper matrix, blue characters) and four nuclear markers (lower matrix, red characters)

Pairwise substitutions four nuclear markers (1628 bp)	Pairwise substitutions in partial mitogenomes (10 PCGs + 12 tRNAs; 8646 bp[a])										
	La[b]	Lp	Ldd_NJ	Ldd_KG	Lda_TJ	Ldj_ID	Ld_IR	Lu_JH	Lx	Lmo	Lma
La		15	526	533	527	523	529	543	786	1190	1391
Lp	3		523	532	524	520	526	541	781	1188	1388
Ldd_NJ	19	22		34	60	56	86	148	693	1106	1349
Ldd_KG	19	22	2		66	62	90	158	714	1118	1363
Lda_TJ	10	23	3	3		10	88	168	712	1122	1365
Ldj_ID	18	21	1	1	2		86	166	707	1120	1358
Ld_IR	20	23	3	3	4	2		152	719	1121	1357
Lu_JH	21	24	4	6	7	5	7		711	1117	1358
Lx	82	84	81	82	83	81	82	83		1088	1284
Lmo	211	211	211	212	213	211	210	214	235		1285
Lma	329	329	328	329	330	328	328	328	348	301	

PCG, protein-coding gene; tRNA, transfer RNA.

[a]The total size of these mt sequences is slightly lower than the total size of the two mitogenome fragments as a result of trimming to remove non-coding or incomplete sequences.

[b]La, *L. albescens*; Lp, *L. postalba*; Ldd_NJ, *L. dispar dispar* USA; Ldd_KG, *L. dispar dispar* Greece; Lda_TJ, *L. dispar asiatica* China; Ldj_ID, *L. dispar japonica* Japan; Ld_IR, *L. dispar* Iran ('Transcaucasian' gypsy moth); Lu_JH, *L. umbrosa* Hokkaido; Lx, *L. xylina*; Lmo, *L. monacha*; Lma, *L. mathura*. Abbreviations for *L. dispar* subspecies are shown in green characters, and pairwise comparisons among them are shown in a grey box. [Colour table can be viewed at wileyonlinelibrary.com].

each with clearly distinct wing patterns and colours (Quek *et al.*, 2010; Hoyal Cuthill & Charleston, 2015). While neutral mt and nuclear markers failed to recover wing-colour morphs in phylogenetic analyses examining intraspecific variation (Brower, 1994, 1996; Flanagan *et al.*, 2004; Quek *et al.*, 2010), a single gene shown to control differences in red wing colour, *optix* (Reed *et al.*, 2011), structured the lineages by red colour patterns (Hines *et al.*, 2011).

Similarly, the data presented here strongly suggest that *L. albescens* and *L. postalba* individuals are members of a single species displaying important wing colour variation, as opposed to being distinct species. Although typical *postalba* moths found on the northern Ryukyu Islands, Kyushu and Shikoku, tend to have wings that are distinctly darker than those of *albescens* moths found in Okinawa and Ryukyu Islands located south of it, a close examination of specimens collected along a transect across the Ryukyu Arc revealed a more or less gradual darkening of the wings as one progresses from south to north, suggesting the occurrence of a wing colour cline, albeit not perfect (Fig. 1; see also Fig. S2). In a comparison of mt and nuclear markers between two specimens collected near the south (*albescens*) and north (*postalba*) ends of the combined *albescens*/*postalba* geographic range, the absence of significant nucleotide variation (Table 3; Fig. 4) indeed supports the conclusion that *albescens* and *postalba* are not different species, but rather represent two distinct wing morphs occurring near the outward geographic limits of the species' range, with specimens found between these extremities typically displaying intermediate wing colour patterns. Phylogenetic analysis of an additional 18 specimens collected along this gradient, using a smaller set of markers (Fig. 5), gave further support to this conclusion.

Our results agree with those of Arimoto & Iwaizumi (2018), who concluded that *L. albescens* and *L. postalba* could not be distinguished from one another on the basis of mt COI and COII markers. Conversely, in another recent study focusing on Japanese gypsy moths, where a third mt marker (ND1) was used for phylogenetic analysis in addition to COI and COII, *L. albescens* was fully resolved from *L. postalba*, with the latter being placed basal to the former (Inoue *et al.*, 2019). However, a careful re-examination of the sequence data used to construct this tree revealed a data-handling error whereby the COI and COII sequences attributed to the *L. albescens* specimens were in fact from *L. dispar* (M. Inoue & V. Martemyanov, unpublished data), effectively producing an arborescence where *L. albescens* was incorrectly placed between *L. postalba* and the *L. dispar* + *L. umbrosa* clade (Inoue *et al.*, 2019).

What we know about the Ryukyu Islands points to the existence of land bridges between most islands at some point in their recent geological history, facilitating insect movement between these land masses. During the period spanning 1.0–0.2 Ma, the islands were all well separated from one another, due to the rise of sea levels (Kimura, 2000). Subsequently (0.2–0.04 Ma), nearly the entire Ryukyu ridge emerged, increasing land areas and forming important land bridges, including one from the Chinese continent. Between 40 000 years ago and now, sea levels rose again and most land bridges disappeared (Kimura, 2000). Thus, for a period of *c.* 160 000 years, there appear to have been limited barriers to the movement of *L. albescens/L. postalba* across the Ryukyu Arc, thereby reducing the potential for reproductive isolation among nascent biotypes. In the more recent past, higher sea levels have created obstacles to insect movement, which could have favoured divergence towards the darker wing form (i.e. *postalba*) on the northernmost islands. However, contemporary human-mediated insect movement between islands is now expected to reduce reproductive isolation among *albescens-postalba* populations. Indeed, there

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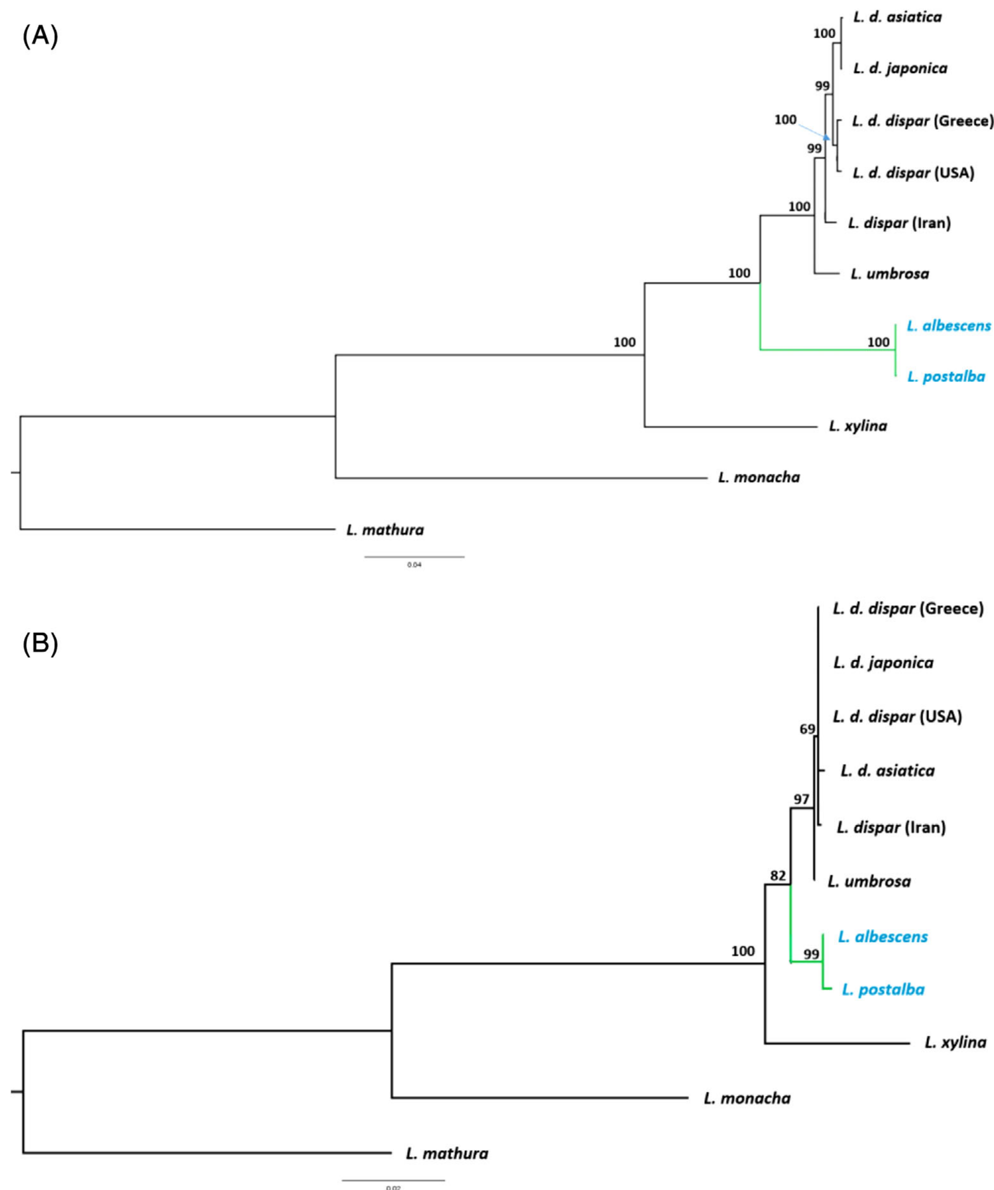

Fig. 4. Phylogenetic relationships among the 11 *Lymantria* populations. (A) Reconstruction based on partial mitogenomes (10 protein-coding genes +12 transfer RNAs). (B) Reconstruction based on four nuclear markers (elongation factor 1-alpha, internal transcribed spacer 2, wingless and ribosomal protein S5). Analyses were conducted using the maximum likelihood method, with 1000 bootstrap replicates, as implemented on the IQ-TREE web server (Trifinopoulos *et al.*, 2016). See Materials and Methods for details on models and partitioning schemes. [Colour figure can be viewed at wileyonlinelibrary.com].

is a well-developed network of ferries between Okinawa and the other Ryukyu islands (https://www.kadenafss.com/wp-content/uploads/2017/02/17-03-Ferry-Schedule.doc), providing a suitable vector for inter-island insect movement.

In conclusion, the data presented in this study indicate that insects described earlier as *L. albescens* and *L. postalba* (Pogue & Schaefer, 2007) form a single species, which we recommend be given the name *L. albescens*. Accordingly, *L. postalba* is relegated to synonymy under *L. albescens* (**syn.n.**). The fact that *postalba* appears to represent a darker version of *L. albescens* at the northern extremity of the forewing colour gradient argues against attributing subspecies status to *postalba*; rather, we propose referring to the darker specimens of *L. albescens* as *postalba* morphs. Whether these morphological differences have a genomic basis is unclear at this stage, given that variation in environmental conditions could also yield different phenotypes, as observed for changes in coloration of *L. dispar* larvae reared under different conditions (V. Martemyanov, personal

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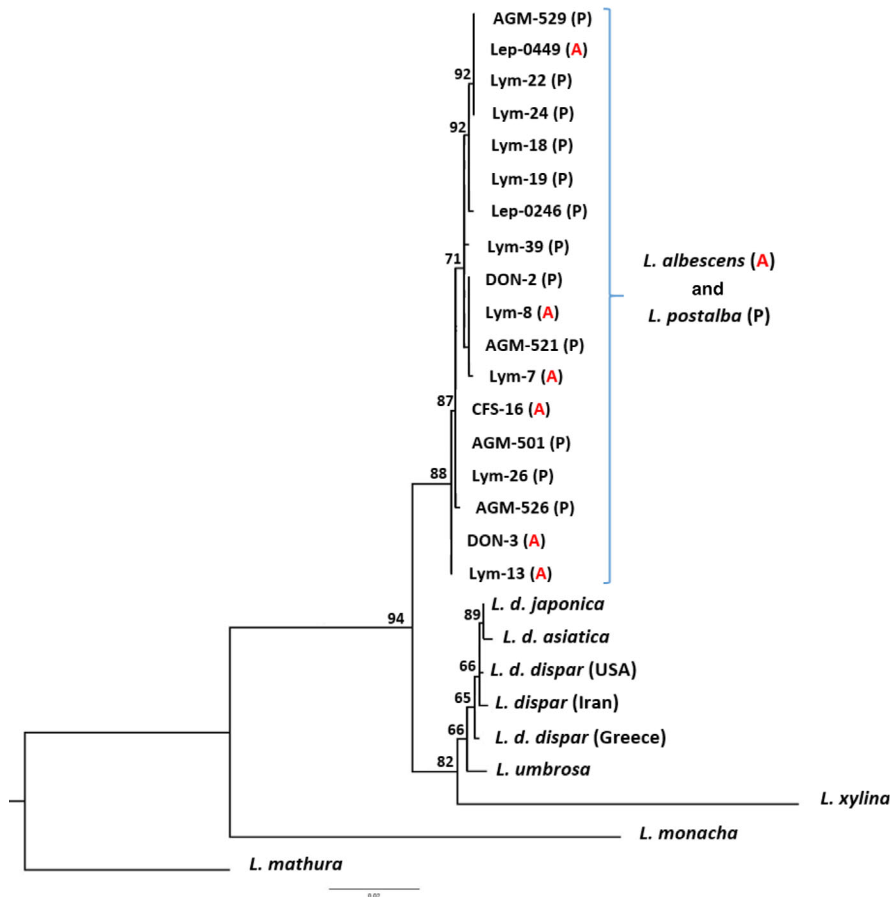

Fig. 5. Phylogenetic relationships among 18 specimens of *Lymantria postalba* (12 specimens) and *Lymantria albescens* (six specimens) collected in their respective range along the Ryukyu Islands, Kyushu and Shikoku (see Fig. 1 for their distribution), together with the additional *Lymantria* taxa and specimens used for constructing the trees shown in Fig. 4. Sequences of one mitochondrial (cytochrome *c* oxydase 1) and two nuclear (elongation factor 1-alpha and wingless) markers were obtained and concatenated for each specimen, and used in a maximum likelihood phylogenetic reconstruction, with 1000 bootstrap replicates, as implemented on the IQ-TREE web server (Trifinopoulos *et al.*, 2016). See Materials and Methods for details on models and partitioning schemes. [Colour figure can be viewed at wileyonlinelibrary.com].

observations). Future studies investigating SNPs across the nuclear genome of *L. albescens* specimens collected over its geographic range may help to identify intraspecific genomic blocks potentially responsible for the observed wing colour variation in this species.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Phylogenetic relationships among the 11 *Lymantria* populations. This tree is based on the concatenation of 10 PCGs +12 tRNAs, from the mitogenome, plus four nuclear gene regions (Ef1α, ITS2, Wgl and RpS5). Analyses were conducted using the maximum likelihood method, with 1000 bootstrap replicates, as implemented on the IQ-TREE web server (Trifinopoulos *et al.*, 2016). See Materials and Methods for details on models and partitioning schemes.

Figure S2. Examples of *L. albescens/postalba* male moths collected across the Ryukyu Arc and Kyushu, along with their collection sites. Numbers on the map refer to individual rows of moths on the picture, which shows an apparent north-to-south gradient in forewing ground colour (background map generated using Google Maps).

Table S1. Primers used for mitochondrial genome amplification and sequencing.

Table S2. Primers used for nuclear/mitochondrial markers amplification and sequencing.

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