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Phylogeography and genetic relationship within *Erebia neriene* (Böber, 1890) in Eastern Asia inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae)

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Abstract

Little is known about the genetic relationships within *Erebia neriene* (Böber, 1890) complexes inhabiting areas outside the Japanese Archipelago. In this study, we investigated genetic variations in *E. neriene* individuals collected from eastern Asia using mitochondrial sequences to reveal the phylogeny and genetic relationships among *E. neriene* complexes inhabiting areas outside the Japanese Archipelago. This study revealed greater genetic differentiation and a clear genetic structure among *E. neriene neriene* and *E. neriene scoparia* (Butler, 1882) populations. This is the first population genetics study of these subspecies.
Keywords: Lepidoptera, Nymphalidae, *Erebia*, COI, gene flow, glaciation, long-distance migration, ND5, Asia.

Filogeografía y relación genética dentro de *Erebia neriene* (Böber, 1890) en Asia oriental inferidas a partir de secuencias de ADN mitocondrial (Lepidoptera: Nymphalidae)

Resumen

Se sabe poco sobre las relaciones genéticas dentro de los complejos de *Erebia neriene* (Böber, 1890) que habitan zonas fuera del archipiélago japonés. En este estudio, investigamos las variaciones genéticas en individuos de *E. neriene* recolectados en Asia oriental utilizando secuencias mitocondriales para revelar la filogenia y las relaciones genéticas entre los complejos de *E. neriene* que habitan zonas fuera del archipiélago japonés. Este estudio reveló una mayor diferenciación genética y una clara estructura genética entre las poblaciones de *E. neriene neriene* y *E. neriene scoparia* (Butler, 1882). Se trata del primer estudio de genética de poblaciones de estas subespecies.
Palabras clave: Lepidoptera, Nymphalidae, *Erebia*, COI, flujo genético, glaciación, migración a larga distancia, ND5, Asia.

Introduction

Erebia neriene (Böber, 1809) is distributed in eastern Asia from Mongolia to Japan. Three subspecies have been recognized: *E. neriene neriene* (Böber 1809), which inhabits continental regions; *E. neriene niphonica* (Janson, 1877), which inhabits Honshu, the mainland of Japan; *E. neriene scoparia* (Butler, 1882), whose

distribution areas are Hokkaido, the northernmost island of Japan, and Sakhalin (Inomata et al. 2010-2013). The habitat of *E. neriene* in the northern region is large, and individuals are commonly observed (e.g., Nagaoka & Xiaoshuan, 2017), whereas *E. neriene niphonica* is restricted to high-altitude areas in the southern region, and each habitat is separated from the other.

The phylogenetic relationships of the subspecies have been investigated using mitochondrial (cytochrome *c* oxidase subunit I, COI, and NADH dehydrogenase subunit 5, ND5) and/or nuclear DNA (wingless) sequences (e.g., Nakatani et al. 2007a; Nakatani et al. 2018). Large genetic differentiation was found among the three subspecies and *E. neriene scoparia* was primarily divided from the others, and the remaining lineages were further divided into *E. nerine niphonica* and *E. neriene neriene*. Additionally, Nakatani et al. (2007b) analyzed the phylogeographic history of Japanese *E. neriene* (i.e. *E. neriene scoparia* inhabiting Hokkaido and *E. neriene niphonica*) and suggested that the clear genetic structure observed within *E. neriene niphonica* might have been influenced by fragmentation and secondary contact due to repeated glaciations.

In contrast to Japanese *E. neriene*, the molecular evaluation of *E. neriene neriene* populations has never been conducted. In addition, because previous studies have focused mainly on the phylogeny of Japanese *E. neriene*, the number of continental samples analyzed were insufficient for robust quantitative assessments. A better understanding of the phylogenetic and genetic relationships requires the expansion of sampling to a broader scale. In this study, we investigated the genetic variations in *E. neriene* individuals collected in eastern Asia using mitochondrial COI and ND5 sequences. The aim of this study was to provide the first phylogeny and genetic relationships among *E. neriene* complexes inhabiting areas outside the Japanese Archipelago (i.e., *E. neriene neriene* and *E. neriene scoparia*). Additionally, sequence data were used to confirm the phylogenetic relationships among the three subspecies.

Materials and methods

We collected 111 specimens of adult *E. neriene* individuals from 11 localities (Honshu, Hokkaido, Moneron, Gornyi, Genhe, and Terelj) between 1999 and 2016 using hand nets (Table I). DNA was extracted from two or three legs using a DNeasy Blood & Tissue Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. Primers C1-J-1718 (Bromilow & Sperling, 2011) and C1-N-2329 (Simon et al. 1994) for COI, and V1 and C2 (Yagi et al. 1999) for ND5 were used for PCR amplification. PCR was performed using KOD FX Neo (TOYOBO, Osaka, Japan) in accordance with the manufacturer's instructions. The thermal conditions were as follows: initial denaturation at 94 ºC for 2 min, 40 cycles of 98 ºC for 10 s, Tm (COI: 46 ºC, ND5: 45 ºC) for 30 s, and 68 ºC for 30 s, final extension at 68 ºC for 5 min, and hold at 10 ºC. The resulting sequences were deposited in DDBJ/EMBL/GenBank with accession numbers LC616315-616359 for COI and LC735100-LC735112 for ND5 (Table II).

Table I. Sampling information of *Erebia neriene* complexes used in this study.

No.	Sample locality	Abbreviation	Subspecies	Year	Long.	Lat.	*N*
1	Japan, Honshu, Iide Mts.	JHI	*niphonica*	2015	37º50.82´N	139º40.93´E	8
2	Japan, Honshu, Takenokosan Mt.	JHT	*niphonica*	2007-2008	36º47.44´N	138º46.10´E	1
3	Japan, Honshu, Tomi Ridge	JHR	*niphonica*	2010	36º39.20´N	137º47.10´E	2
4	Japan, Honshu, Onyu Pond	JHO	*niphonica*	2010	36º08.17´N	137.32.59´E	1
5	Japan, Honshu, Sanpuku Mountain Pass	JHS	*niphonica*	2007	35º33.14´N	138º08.75´E	3
6	Japan, Hokkaido, Samani	HOS	*scoparia*	2011	45º05.03´N	134º02.59´E	10
7	Japan, Hokkaido, Wakkanai	HOW	*scoparia*	1999	45º31.06´N	141º56.46´E	1
8	Russia, Moneron Is.	RUM	*scoparia*	2010, 2013	46º16.88´N	141º12.46´E	29
9	Russia, Gornyi	RUG	*neriene*	2015	50º46.42´N	136º25.06´E	22
10	Mongolia, Terelj	MOT	*neriene*	2016	47º56.07´N	107º23.50´E	12
11	China, Genhe	CHG	*neriene*	2016	50º49.39´N	121º38.79´E	22

Table II. Haplotype composition and represented samples of each concatenated haplotype. For abbreviations, see Table 1.

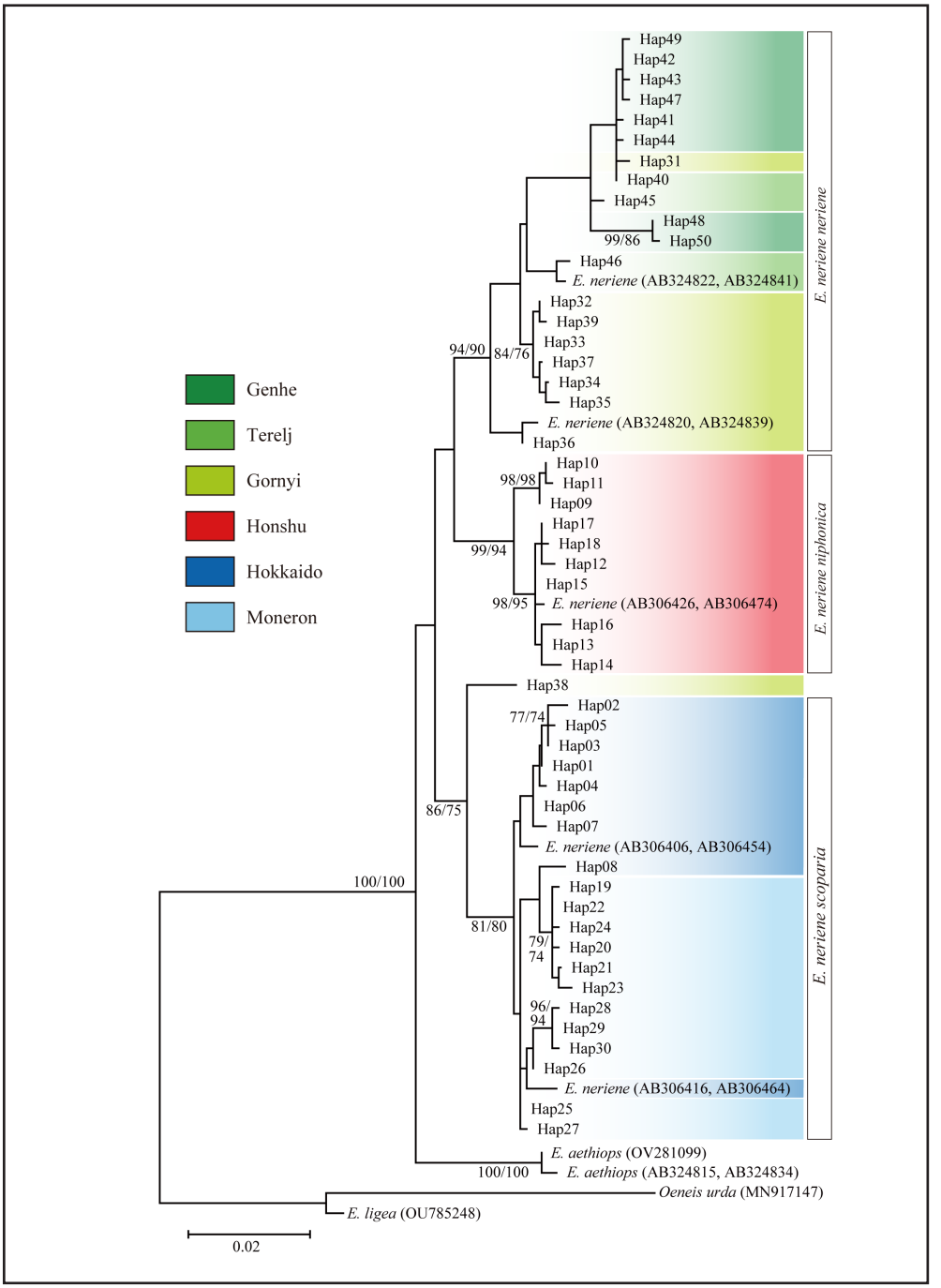
Haplotype name of COI	Accession no. of COI	Haplotype name of ND5	Accession no. of ND5	Haplotype name of total sequence	Haplotype represented (*N*)
COI Hap1	LC616315	ND5 Hap1	LC735100	Hap1	HOS (2) JHI (1)
COI Hap2	LC616316	ND5 Hap1		Hap2	HOS (1)
COI Hap3	LC616317	ND5 Hap1		Hap3	HOS (2)
COI Hap4	LC616318	ND5 Hap1		Hap4	HOS (1)
COI Hap5	LC616319	ND5 Hap1		Hap5	HOS (1)
COI Hap6	LC616320	ND5 Hap1		Hap6	HOS (2)
COI Hap7	LC616321	ND5 Hap1		Hap7	HOS (1)
COI Hap8	LC616322	ND5 Hap2	LC735101	Hap8	HOW (1)
COI Hap9	LC616323	ND5 Hap3	LC735102	Hap9	JHI (4)
COI Hap10	LC616324	ND5 Hap3		Hap10	JHI (2)
COI Hap11	LC616325	ND5 Hap3		Hap11	JHI (1)
COI Hap12	LC616326	ND5 Hap4	LC735103	Hap12	JHT (1)
COI Hap13	LC616327	ND5 Hap5	LC735104	Hap13	JHR (1)
COI Hap14	LC616328	ND5 Hap5		Hap14	JHR (1)
COI Hap15	LC616329	ND5 Hap5		Hap15	JHO (1)
COI Hap16	LC616330	ND5 Hap5		Hap16	JHS (1)
COI Hap17	LC616331	ND5 Hap5		Hap17	JHS (1)
COI Hap18	LC616332	ND5 Hap5		Hap18	JHS (1)
COI Hap19	LC616333	ND5 Hap6	LC735105	Hap19	RUM (12)
COI Hap20	LC616334	ND5 Hap7	LC735106	Hap20	RUM (1)
COI Hap21	LC616335	ND5 Hap6		Hap21	RUM (1)
COI Hap20	LC616334	ND5 Hap6		Hap22	RUM (4)
COI Hap22	LC616336	ND5 Hap6		Hap23	RUM (1)
COI Hap23	LC616337	ND5 Hap6		Hap24	RUM (1)
COI Hap24	LC616338	ND5 Hap6		Hap25	RUM (2)
COI Hap25	LC616339	ND5 Hap6		Hap26	RUM (1)
COI Hap26	LC616340	ND5 Hap6		Hap27	RUM (1)
COI Hap28	LC616342	ND5 Hap6		Hap28	RUM (2)
COI Hap27	LC616341	ND5 Hap6		Hap29	RUM (2)

COI Hap27	LC616341	ND5 Hap7		Hap30	RUM (1)
COI Hap29	LC616343	ND5 Hap8	LC735107	Hap31	RUG (1)
COI Hap30	LC616344	ND5 Hap9	LC735108	Hap32	RUG (7)
COI Hap31	LC616345	ND5 Hap9		Hap33	RUG (5)
COI Hap32	LC616346	ND5 Hap9		Hap34	RUG (1)
COI Hap33	LC616347	ND5 Hap9		Hap35	RUG (1)
COI Hap34	LC616348	ND5 Hap10	LC735109	Hap36	RUG (1)
COI Hap35	LC616349	ND5 Hap9		Hap37	RUG (1)
COI Hap36	LC616350	ND5 Hap10		Hap38	RUG (1)
COI Hap37	LC616351	ND5 Hap9		Hap39	RUG (4)
COI Hap38	LC616352	ND5 Hap8		Hap40	MOT (10) CHG (3)
COI Hap39	LC616353	ND5 Hap8		Hap41	MOT (1)
COI Hap38	LC616352	ND5 Hap11	LC735110	Hap42	CHG (8)
COI Hap38	LC616352	ND5 Hap12	LC735111	Hap43	CHG (1)
COI Hap38	LC616352	ND5 Hap13	LC735112	Hap44	CHG (1)
COI Hap40	LC616354	ND5 Hap9		Hap45	MOT (1)
COI Hap41	LC616355	ND5 Hap11		Hap46	CHG (1)
COI Hap42	LC616356	ND5 Hap11		Hap47	CHG (1)
COI Hap43	LC616357	ND5 Hap10		Hap48	CHG (2)
COI Hap44	LC616358	ND5 Hap11		Hap49	CHG (1)
COI Hap45	LC616359	ND5 Hap10		Hap50	CHG (4)

Neighbor-joining (NJ) and maximum likelihood (ML) trees were constructed with concatenated mtDNA sequences of COI and ND5 using MEGA 11 (Tamura et al. 2021). The p-distance model was used for NJ analysis. Prior to ML phylogenetic estimation, the best nucleotide substitution model was evaluated using MEGA 11, and the HKY + G model was selected based on corrected Akaike information criterion (AICc) scores. The robustness of the nodes in each tree was assessed by generating 1,000 bootstrap replicates. For the phylogenetic analysis, five additional sequences of *E. neriene* (see Figure 1) reported by Nakatani et al. (2007a) were included as references. The following sequences from related species were used as outgroups: *E. aethiops* (Esper, 1777) (accession numbers: OV281099, AB324815 and AB324834), *E. ligea* (Linnaeus, 1758) (OU785248), and *Oeneis urda* (Eversmann, 1847) (MN917147). The lack of nucleotides in some of the cited data was treated as missing.

We calculated the extent of genetic differentiation using Φ_{ST} (Michalakis & Excoffier, 1996) and Nei's average number of differences (D_A; Nei & Li, 1979) to infer genetic relationships among populations. We focused on the genetic differentiation among *E neriene* populations inhabiting areas outside of Japan in this analysis; thus, we pooled samples collected from two localities in Hokkaido and from five in Honshu as Hokkaido and Honshu, respectively. The statistical significance of these tests was obtained using 10,000 permutations in Arlequin 3.5 (Excoffier & Lischer, 2010) and corrected after the false discovery rate (FDR; Benjamini & Yekutieli, 2001).

Figure 1. Maximum likelihood (ML) tree of *Erebia neriene* based on the concatenated sequence of COI (624 bp) and ND5 sequences (386 bp). Numbers on the nodes represent NJ (left) and ML (right) bootstrap values (>70%, n = 1000).



Results

Based on the 624 bp COI (45 haplotypes) and 386 bp ND5 sequences (13 haplotypes), 50 haplotypes were identified among the 111 *E. neriene* individuals (Table II). No insertion/deletion mutations were identified in the sequences. The distribution of haplotypes showed a strong genetic structuring, and only two haplotypes were observed at different localities (Hap1 and Hap40; Table II).

The ML tree revealed three major clades of *E. neriene* inhabiting Eastern Asia, each of which corresponded to the haplotypes *E. neriene neriene*, *E. neriene niphonica*, and *E. neriene scoparia* (Figure 1). The *E. neriene scoparia* clade was primarily divided from the others, and the remaining haplotypes were further separated into *E. neriene niphonica* and *E. neriene neriene.* Except for one haplotype observed in Gornyi (Hap38), the haplotypes observed in each subspecies formed a monophyletic clade. In *E. neriene neriene*, the haplotypes observed in each population did not form a monophyletic clade despite their geographical distance, indicating dispersal and/or gene flow among these continental regions. The topology of the NJ tree was similar to that of the ML trees, except that the *E. aethiops* clade formed a monophyletic clade with *E. neriene neriene* and *E. neriene niphonica* clades.

For pairwise comparison, the Φ_{ST} and D_A values ranged from 0.1376 to 0.9363 and 0.9740 to 31.8788, respectively (Table III). Significant genetic differentiation was observed within all comparisons of *E. neriene* populations except for one comparison (i.e., between Terelj and Genhe).

Table III. Pairwise Φst (below) and D_A (above diagonal) among regional populations of *Erebia neriene* complexes.

-	Honshu	Hokkaido	Moneron	Gornyi	Terelj	Genhe
Honshu		**20.2026**	**22.3967**	**16.9039**	**28.1238**	**24.4675**
Hokkaido	**0.7557**		**5.4259**	**21.5085**	**31.8788**	**28.5608**
Moneron	**0.7832**	**0.5454**		**24.1638**	**30.2890**	**26.9140**
Gornyi	**0.7039**	**0.8074**	**0.8180**		**13.6255**	**12.0527**
Terelj	**0.8373**	**0.9363**	**0.8867**	**0.7575**		0.9740
Genhe	**0.7530**	**0.8225**	**0.8171**	**0.6411**	0.1376	-
bold-p<0.001after FDR (B-Y) method						

Discussion

The populations of *E. neriene* complexes did not share any haplotype except for two (haplotypes 1 and 40), and greater genetic differentiation was observed even among neighboring populations, such as between Moneron and Hokkaido. These results indicate that *E. neriene* inhabiting different regions is independent, and gene flow is rare. These endemic genetic features are attributed to glaciation. Populations inhabiting areas strongly influenced by glaciation are likely to show greater genetic differentiation and uniqueness (Schmitt et al. 2006; Schmitt & Haubrich, 2008). It has been reported that Eastern Asia may be influenced by glacial-interglacial cycles (Frenzel, 1968). During glacial periods, *E. neriene neriene* populations in the continental region may repeatedly undergo depopulation, probably due to low temperatures and subsequent recolonization, resulting in genetic bottlenecks and subsequent large genetic differentiation. However, *E. neriene scoparia* and *E. neriene niphonica* populations may have been separated mainly by transgression or decreasing habitats caused by high temperatures during the interglacial period (Nakatani et al. 2007b). Thus, a strong genetic structure was observed.

Although the genetic uniqueness of each population was observed, Terelj and Genhe did not show significant genetic differentiation despite their geographical distance, and the haplotypes of these populations formed a monophyletic clade. There is a vast habitat area suitable for *E. neriene neriene* in these areas, and *E. neriene neriene* is distributed over a wide range of habitats (e.g., Nagaoka & Xiaoshuan 2017). In addition, there are no obvious barriers to dispersal, such as oceans, mountains, or deserts, between Terelj and Genhe. Therefore, the habitat areas of Terelj and Genhe could have been connected, and these areas might have acted as large populations rather than distinct habitats.

However, the fact that some haplotypes found in Gornyi (i.e., haplotypes 31 and 38) and Hokkaido (haplotype 8) formed the same cluster as those found in different populations may be due to long-distance migration. Gornyi, Moneron, and Hokkaido are separated from other regions by mountains and oceans. Superior migration ability has been reported in some Papilionoidea (e.g., *Parantica sita* (Kollar, 1844)), which can migrate thousands of kilometers (Honda et al. 2016). Therefore, *E. neriene* may conduct long-distance migration, even across the ocean, and has superior migration ability.

The phylogenetic tree observed in this study also showed that the haplotypes of *E. neriene scoparia* were primarily divided from those of the other two subspecies and that *E. neriene scoparia* and *E. neriene niphonica* immigrated separately from the continental region to Japan. This result supports those of previous studies (e.g., Nakatani et al. 2007a; Nakatani et al. 2018; Sekiguchi et al. 2002) and indicates that greater genetic differentiation could be a genetic feature of *E. neriene* complexes.

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Conflict of Interest

The authors declare that they have no known financial interest or personal relationship that could have influence the work presented in this article.

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