Entomological Science, 2000, 3(2): 321–329

Phylogeny of *Luehdorfia* Butterflies Inferred from Mitochondrial ND5 Gene Sequences

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Abstract. The phylogeny of the genus *Luehdorfia* (Lepidoptera, Papilionidae) in the East Asia was examined by a molecular approach. To elucidate the phylogenetic relationships among four species of the genus, partial nucleotide sequences (785 base pairs) of the mitochondrial NADH dehydrogenase subunit 5 (ND5) gene were compared. The results indicated that *Luehdorfia japonica* diverged initially from the ancestral stock of the genus *Luehdorfia,* and that then the ancestral stock successively yielded *L. chinensis, L. longicaudata* and *L. puziloi.* These findings suggest that *L. japonica* might immigrate first among the *Luehdorfia* butterflies into the area of the current Japanese Archipelago after the intrusion of *Asarum* as the food plant of *L. japonica.* The phylogeny of the genus Luehdorfia is discussed.

Key words: mitochondrial DNA, ND5 gene, phylogeny, *Luehdorfia,* Aristolochiaceae, biogeography.

Introduction

The genus *Luehdorfia*, which belongs to the tribe Zerynthiini (Papilionidae, Parnassiinae), is composed of four species: *Luehdorfia japonica* Leech, *Luehdorfia chinensis* Leech, *Luehdorfia longicaudata* Lee and *Luehdorfia puziloi* (Erschoff). *L. puziloi* is further classified into four subspecies: *L. p. puziloi* (Erschoff), *L. p. coreana* Matsumura, *L. p. inexpecta* Sheljuzhko and *L. p. yessoensis* Rothschild. Both *L. japonica* and *L. puziloi* which are familiar to the Japanese people, have been studied from various aspects: life history, wing patterning, distribution, species-specific host plants (Takahashi, 1973; Hiura, 1978; Shinkawa, 1991; Honda *et al.*, 1995), morphologies of larvae or imagines (Saigusa, 1973; Honda, 1980; Ishizuka, 1980; Shinkawa, 1996), interspecific cross-breedings (Ishizuka, 1991; Kudoh & Inaoka, 1995), chromosomics (Saitoh, 1988) and isozyme analyzes (Kominami, 1987).

The imagines of various species of the genus *Luehdorfia* emerge in early spring simultaneously with the germination of the host plants and the larvae feed on species-specific host plants. The distribution of each species of *Luehdorfia* is consistent with that of their host plants of the family Aristolochiaceae (Fig. 1). *L. chinensis* is distributed in the mid-southern China together with the food plant, *Asiasarum forbesii,* and *L. longicaudata* is distributed in the mountainous regions of mid-southern China together with the food plant, *Saruma henryi. L. puziloi* is distributed in the Korean Peninsula (*L. p. coreana*), the southeastern Russia (*L. p. puziloi*) and the Japanese Archipelago (*L. p. inexpecta* and *L. p. yessoensis*) in accordance with the distribution of host plants belonging to the genus *Asiasarum. L. japonica* is endemic to Japan and distributed in the central and western parts of Honshu together with the food plants of *Asarum*. Takahashi (1973) and Hiura (1978) suggested that *L. japonica* diverged from *L. p. inexpecta* as a result of host alteration from the deciduous genus *Asiasarum* to the evergreen genus *Asarum*, based on the fact that *L. japonica* feeds on plants of *Asiasarum* in some cases.

In the present study, we examine the nucleotide sequences of the mitochondrial gene encoding ND5 of *Luehdorfia* butterflies to explore the evolution and phylogeny of the genus. The ND5 gene shows a fairly rapid evolution and is one of the most useful mitochondrial genes for investigating the phylogenetic

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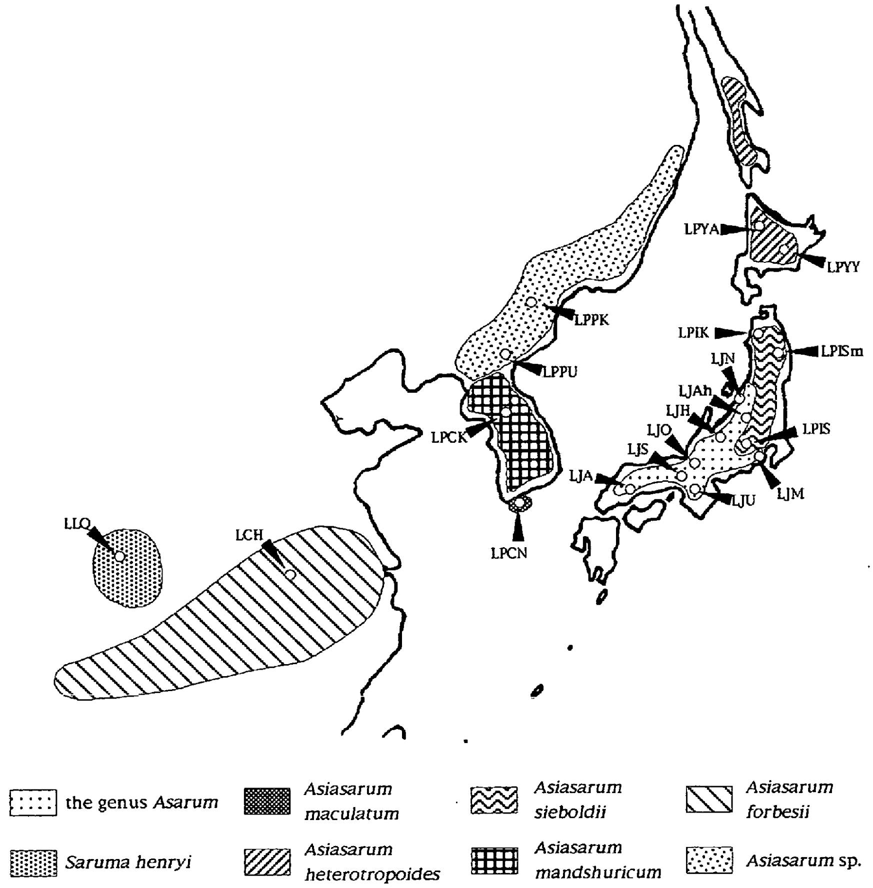

Fig. 1. Map showing the geographical distribution of the family of Aristolochiaceae and the sampling localities of the genus *Luehdorfia*. The distribution of the family of Aristolochiaceae is represented in patterns marked in the eight columns beneath the map. Because a few *Asiasarum* species are distributed in southeastern Russia, we refer to the populations of southeastern Russia as *Asiasarum* sp. Locality codes correspond to sample codes in Table 1. Each species of *Luehdorfia* is distributed consistent with the distribution of the species-specific host plants of the family of Aristolochiaceae.

relationships among related groups (Su *et al.*, 1996a, b, c).

Materials and Methods

Samples and DNA extraction

Species and sources of butterflies examined in this study are shown in Table 1. For the phylogenetic analysis of *Luehdorfia*, the genus *Bhutanitis*, *Bhutanitis mansfieldi* and *B. thaidina* were used as outgroups. To avoid DNA degradation, all butterfly samples were killed immediately after collection, immersed in 95% ethanol and stored at −20℃ until use. DNA was extracted from the thorax muscle according to the method of Su *et al.* (1996a) with using of a modified proteinase solution consisted of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1% SDS and 0.1 g/m*l* proteinase K (Sigma Chemical Co., St. Louis, MO, USA).

PCR amplification

Extracted total DNA was used as a template for mitochondrial ND5 gene amplification by polymerase chain reaction (PCR) (Saiki *et al.*, 1988). The PCR primers were referred according to the method of Su *et al.* (1996a) and Yagi *et al.* (1999). PCR amplification was carried out in 50 μl of solution containing 1 μg of template DNA, 1 pmol/μl of each primer, and 1.25 U *TaKaRa Ex Taq* (Takara Shuzo Co., Kyoto, Japan). The amplification comprised 35 cycles of denaturation at 94℃ for 30 sec, primer annealing at 46℃ for 30 sec and primer extension at 72℃ for 1 min.

Direct sequencing of PCR-amplified single stranded DNAs

The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Direct sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Lincoln, USA). The nucleotide sequences were determined using a

Table 1. Codes and sources of *Luehdorfia* and other Papilionidae butterflies

Species	Code	No.	Source	DDBJ No.
Luehdorfia japonica	LJN	2 (LJN1,2)	Nagaoka, Niigata, Japan	AB016825
	LJH	1 (LJH1)	Hakuba, Nagano, Japan	*[1]
	LJO	1 (LJO1)	Ohno, Gifu, Japan	*[1]
	LJAh	1 (LJAh1)	South Aizu, Fukushima, Japan	*[1]
	LJU	1 (LJU1)	Ueno, Mie, Japan	AB016827
	LJM	1 (LJM1)	Miyagase, Kanagawa, Japan	AB029993
	LJA	1 (LJA1)	South Asa, Hiroshima, Japan	*[2]
	LJS	1 (LJS1)	Sakyo, Kyoto, Japan	*[2]
Luehdorfia chinensis	LCH	3 (LCH1, 2, 3)	Hangzhou, Zhejiang, China	AB016826
Luehdorfia longicaudata	LLQ	4 (LLQ1, 2, 3, 4)	Mt. Qin Ling in Xian, Shanxi, China	AB016828
Luehdorfia puziloi coreana	LPCK	1 (LPCK1)	Hwa San, Kyonggido, Korea	AB016830
	LPCN	3 (LPCN1, 2, 3)	Nam Hae, Kyongsangnamdo, Korea	*[3]
Luehdorfia puziloi inexpecta	LPIS	2 (LPIS1, 2)	Sutama, Yamanashi, Japan	AB016832
		1 (LPIS3)	Sutama, Yamanashi, Japan	AB029992
	LPIK	1 (LPIK1)	Kumotani, Aomori, Japan	AB016833
	LPISm	1 (LPISm1)	Sendai, Miyagi, Japan	*[4]
Luehdorfia puziloi yessoensis	LPYA	1 (LPYA1)	Atsuta, Hokkaido, Japan	AB016831
	LPYY	2 (LPYY1, 2)	Yubari, Hokkaido, Japan	*[5]
Luehdorfia puziloi puziloi	LPPK	3 (LPPK1, 2, 3)	Khabarovsk, Primorsky, Russia	AB016829
	LPPU	2 (LPPU1, 2)	Uladivostok, Primorsky, Russia	*[6]
Bhutanitis mansfieldi	—	1	Liangshan Yizu, Sichuan, China	AB026727
Bhutanitis thaidina	—	1	Northwest Yunnan, China	AB026728

*[1] This sequence is the same as AB016825.
*[2] This sequence is the same as AB029993.
*[3] This sequence is the same as AB016830.
*[4] This sequence is the same as AB016833.
*[5] This sequence is the same as AB016831.
*[6] This sequence is the same as AB016829.

ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

Phylogenetic analysis

Phylogenetic analysis of the sequences amplified from the ND5 genes was performed by three methods: the neighbor-joining (NJ) method (Saitou & Nei, 1987), the unweighted pair-group method using an arithmetic average (UPGMA, Sokal & Michener, 1958) and maximum parsimony (MP) method. The neighbor-joining method with a bootstrap test (Felsenstein, 1985) was performed using the CLUSTAL X program (Thompson *et al.*, 1997). The UPGMA method with a bootstrap test was done by the PHYLIP software package Ver. 3.572c (Dnadist–Neighbor–Seqboot–Consense, Felsenstein, 1993). The evolutionary distances were computed by Kimura's two-parameter method (Kimura, 1980). The maximum parsimony method with a bootstrap test was done by the PAUP* 4.0 (Swofford, 1993).

Results

Nucleotide sequences

The partial sequences (785 bases) of the mitochondrial ND5 genes of the *Luehdorfia* butterflies and two *Bhutanitis* butterflies, *B. mansfieldi* and *B. thaidina*, as outgroups, were determined (Fig. 2). There were neither insertions nor deletions. The nucleotide sequence data of the *Luehdorfia* butterflies have been deposited in the DDBJ database with the accession numbers: AB016825–AB016833, AB020339–AB020340 and AB029992–AB029993.

Genetic variations

The sequence differences and numbers of substitutions obtained from all pairwise comparisons are shown in Table 2. Intraspecific (or intrasubspecific) variations in the nucleotide sequences were observed in *L. japonica* and *L. p. inexpecta*. The *Luehdorfia* butterflies were clearly distinct from the outgroup species, *Bhutanitis mansfieldi* and *B. thaidina*, with 10.83–12.87% (85–101/785 bases) sequence differ-

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                         10        20        30        40        50        60        70
L. japonica 1    GATTCGTTTTAATTTTATATTAATTGATATATTTTTTTTAAAAATTTTATTATTATTATCAGGTTTAACT
L. japonica 2    ······································································
LJU              ······································································
L. longicaudata  A·····································································
L. chinensis     A·················G··················A·······C························
LPPK             A·····G···············································G···············
LPPU             A·····G···············································G···············
LPCK             A·····G···············································G···············
LPCN             A·····G···············································G···············
LPIS1-2          A·····G···············································G···············
LPIS3            A·····G···············································G···············
LPIK             A·····G···············································G···············
L. p. yessoensis A·····G···············································G···············
B. mansfieldi    A·····A········A········A······································A······
B. thaidina      ······A······G·A········A·········A····G···T···················A·····A

                         80        90       100       110       120       130       140
L. japonica 1    ATAATAATAGCTGGAATTTCTGCTAATTATGAATTTGATTTAAAAAAAATTATTGCTTTATCTACTTTAA
L. japonica 2    ······································································
LJU              ······································································
L. longicaudata  ···············G·········T············································
L. chinensis     ···············G······················································
LPPK             ···T···········G······················································
LPPU             ···T···········G······················································
LPCK             ···T···········G······················································
LPCN             ···T···········G······················································
LPIS1-2          ···T···········G·····················································G·
LPIS3            ···T···········G·····················································G·
LPIK             ···T···········G·····················································G·
L. p. yessoensis ···T···········G·····················································G·
B. mansfieldi    ···T·T············AGA··························G··············A·······
B. thaidina      ···T·T········G·····A·········································A·······

                        150       160       170       180       190       200       210
L. japonica 1    GTCAATTAGGCTTAATAATAAGTATTTTAAGAATAGGTTTATCTGATTTAGCTTTTTTTCATTTATTAAC
L. japonica 2    ······································································
LJU              ··········T···························································
L. longicaudata  ·······G··T···········A········G······································
L. chinensis     ·······G··T···········A········G··········T···························
LPPK             ·······G··T···········A········G······································
LPPU             ·······G··T···········A········G······································
LPCK             ·······G··T···········A········G······································
LPCN             ·······G··T···········A········G······································
LPIS1-2          ·······G··T···········A········G······································
LPIS3            ·······G··T···········A········G······································
LPIK             ·······G··T···········A········G······································
L. p. yessoensis ·······G··T···········A········G······································
B. mansfieldi    ·A········T···········A··················G·A··························
B. thaidina      ·A········T···························A·····A··························

                        220       230       240       250       260       270       280
L. japonica 1    TCATGCTATATTTAAAGCTTTATTATTTATATGTTCAGGAGTTATTATTCATATGATAAATGATATTCAA
L. japonica 2    ······································································
LJU              ······································································
L. longicaudata  ···············G······································A···············
L. chinensis     ···············G······································A···············
LPPK             ····································G··G··············A···············
LPPU             ····································G··G··············A···············
LPCK             ····································G··G··············A···············
LPCN             ····································G··G··············A···············
LPIS1-2          ····································G··G··············A···············
LPIS3            ····································G··G··············A···············
LPIK             ····································G··G··············A···············
L. p. yessoensis ····································G··G··············A···············
B. mansfieldi    A·································G·T··············C··A······A········
B. thaidina      A·······················G·········G·T···A·A···········A····G··········

                        290       300       310       320       330       340       350
L. japonica 1    GATATTCGTTTTATGGGAGGAATTAATTTATATATTCCTTTAACTTCCTTATGTATAAATATTTCTAATT
L. japonica 2    ······································································
LJU              ······································································
L. longicaudata  ········G·····A··G·····························T·····C················
L. chinensis     ········G·····A··G···········G·················T······················
LPPK             ··············A··T·····························T·····················C
LPPU             ··············A··T·····························T·····················C
LPCK             ··············A··T·····························T······················
LPCN             ··············A··T·····························T······················
LPIS1-2          ··············A··T·····························T·····················C
LPIS3            ··············A··T·····························T·····················C
LPIK             ··············A··T·····························T·····················C
L. p. yessoensis ··············A··T·····························T·····················C
B. mansfieldi    ··········A···A··T·······GA···A················T······T···············
B. thaidina      ··············A··G···T·G·GA····················A······T···············

                        360       370       380       390       400       410       420
L. japonica 1    TAGCTTTATGTGGTATTCCTTATTTAGCTGGATTTTACTCTAAAGATTTAATTTTAGAAATAGTTAGAAT
L. japonica 2    ······································································
LJU              ······································································
L. longicaudata  ·····································T·····G··························
L. chinensis     ·····································T·····G··························
LPPK             ·····················T···············T································
LPPU             ·····················T···············T································
LPCK             ·····················T···············T································
LPCN             ·····················T···············T································
LPIS1-2          ·····································T································
LPIS3            ·····································T································
LPIK             ················C····················T································
L. p. yessoensis ·····································T································
B. mansfieldi    ··T··················T·········G·····T··G·················T·······T···
B. thaidina      ·············A·······T······G········T·················G··············
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Fig. 2. Alignment of mitochondrial NADH dehydrogenase subunit 5 gene sequences from the *Luehdorfia* and two other Bhutanitis butterflies. The species codes refer to those in Table 1, but *L. japonica* 1 (LJN, LJH, LJO and LJAh), *L. japonica* 2 (LJM, LJA and LJS), *L. p. puziloi* (LPPK and LPPU), *L. p. coreana* (LPCK and LPCN), *L. p. yessoensis* (LPYA and LPYY) and *L. p. inexpecta* (LPIK and LPISm) show no intraspecific sequences variation in each species. Dots indicate nucleotides identical with those in *L. japonica*. Neither insertions nor deletions are detected.

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                         430       440       450       460       470       480       490
L. japonica 1     AAGAAATTTAAATATAATAATTTTTTTTTTATATTATTTTTCAACTGGATTGACTATATTTTATACTATT
L. japonica 2     ······································································
LJU               ······································································
L. longicaudata   ···················································A··················
L. chinensis      ···················································A··················
LPPK              ···G···············································A··················
LPPU              ···G···············································A··················
LPCK              ···G···············································A··················
LPCN              ···G···············································A··················
LPIS1-2           ···G···············································A··················
LPIS3             ···G···············································A··················
LPIK              ···G···············································A··················
L. p. yessoensis  ···G···············································A··················
B. mansfieldi     ··AT·······················A·········A····T·····T··A··A··G··C·········
B. thaidina       ·············T·············A·········A····T·····T··A··AT···A··········

                         500       510       520       530       540       550       560
L. japonica 1     CGTTTATTAATATATTTAATAATTAATGATTATAATTTATTAACAATTTATAATTTATATGATGAAGATT
L. japonica 2     ······································································
LJU               ······································································
L. longicaudata   ·················G···············································G····
L. chinensis      ·················G·······················G·······················G····
LPPK              ··············································································C·······
LPPU              ··············································································C·······
LPCK              ··············································································C·······
LPCN              ··············································································C·······
LPIS1-2           ······································································
LPIS3             ··············································································C·······
LPIK              ··············································································C·······
L. p. yessoensis  ··············································································C·······
B. mansfieldi     ··AA··A························T·····················A················
B. thaidina       ···A··A··········G···G·················A··GT·········A················

                         570       580       590       600       610       620       630
L. japonica 1     ATATTATGTTAAAGAGAATATTTATATTATTATTAATAAGATTAATTTCTGGAAGGTTTTTGATGTGATT
L. japonica 2     ······································································
LJU               ······································································
L. longicaudata   ·······A·····A·································A·······A·····A··A·····
L. chinensis      ·······A·····A·································A·······A·····A··A·····
LPPK              ·······A·····A·········T········A····G·········A·······A·····A··A·····
LPPU              ·······A·····A·········T········A····G·········A·······A·····A··A·····
LPCK              ·······A·····A·········T········A····G·········A·······A·····A··A·····
LPCN              ·······A·····A·········T········A····G·········A·······A·····A··A·····
LPIS1-2           ·······A·····A·········T········A····G·········A·······A·····A··A·····
LPIS3             ·······A·····A·········T········A····G·········A·······A·····A··A·····
LPIK              ·······A·····A·········T········A····G·········A·······A·····A··A·····
L. p. yessoensis  ·······A·····A·········T········A····G·········A·······A·····A··A·····
B. mansfieldi     T······A··G··A······A·AT··········T··G······G·AGTA·····A·····A··A···A·
B. thaidina       ··G····A··G··A·········T··A·······T··········CAGTA·····A·····A······A·

                         640       650       660       670       680       690       700
L. japonica 1     AATTTTTAATTATCCTTATATAATTTATTTACCTTTTAATTTAAAAATATTAGTTATTTATGTAAGATTG
L. japonica 2     ·····································································A
LJU               ······································································
L. longicaudata   ········G············G·········································T···A·A
L. chinensis      ········G············G·········································T···A·A
LPPK              ········G····················································A·T······
LPPU              ········G····················································A·T······
LPCK              ········G····················································A·T······
LPCN              ········G····················································A·T······
LPIS1-2           ········G····················································A·T······
LPIS3             ········G····················································A·T······
LPIK              ········G····················································A·T······
L. p. yessoensis  ········G····················································A·T······
B. mansfieldi     ············C······················A··········T··A····A······A····T··A
B. thaidina       ·····················G·············A··········T··········A·····G··T··T

                         710       720       730       740       750       760       770
L. japonica 1     ATAGGTTTATTAATAGGGTATTTAATTAGAAATATAAAAATTTATAGTTTAAATAAATTTTTAATAACTT
L. japonica 2     ······································································
LJU               ···············································G······················
L. longicaudata   ···········T·····A······································G·············
L. chinensis      ···········T·····A····················································
LPPK              ···········T·····················································G····
LPPU              ···········T·····················································G····
LPCK              ···········T·····················································G····
LPCN              ···········T·····················································G····
LPIS1-2           ···········T·····················································G····
LPIS3             ···········T·····················································G····
LPIK              ···········T·····················································G····
L. p. yessoensis  ···········T·····················································G····
B. mansfieldi     T····A··T·····A··T···················G·······TCGG·T············T···T··
B. thaidina       ·····A··TA·T·····A·····GG·A···G··············TCAA·T············T···T··

                         780  785
L. japonica 1     ATAGTTTAAGAAATT
L. japonica 2     ···············
LJU               ···············
L. longicaudata   ···A···········
L. chinensis      ···A···········
LPPK              ···A·A·········
LPPU              ···A·A·········
LPCK              ···A·A·········
LPCN              ···A·A·········
LPIS1-2           ···A·A·········
LPIS3             ···A·A·········
LPIK              ···A·A·········
L. p. yessoensis  ···A·A·········
B. mansfieldi     ···A···········
B. thaidina       ···A······C····
```

Fig. 2. Continued.

Table 2. Percent differences (above diagonal) and difference numbers (below diagonal) for mitochondrial ND5 sequences (785 bases).

Species (Code[a])	*L. jap* 1	*L. jap* 2	LJU	*L. long.*	*L. chin.*	*L. p. puz.*	*L. p. cor.*	*L. p. yes.*	LPIS	LPIS 3	LPIK	*B. mans.*	B. thai.
L. japonica 1[b]	—	0.13	0.25	4.33	4.71	4.71	4.59	4.71	4.59	4.71	4.84	11.85	12.36
L. japonica 2[b]	1	—	0.38	4.20	4.59	4.84	4.71	4.84	4.71	4.84	4.97	11.72	12.36
L. japonica (LJU)	2	3	—	4.33	4.71	4.71	4.59	4.71	4.59	4.71	4.84	11.59	12.23
L. longicaudata	34	33	34	—	1.15	3.57	3.44	3.57	3.44	3.57	3.69	11.97	12.10
L. chinensis	37	36	37	9	—	3.95	3.82	3.95	3.82	3.95	4.08	12.36	12.48
L. p. puziloi[b]	37	38	37	28	31	—	0.13	0.25	0.38	0.25	0.38	10.96	12.48
L. p. coreana[b]	36	37	36	27	30	1	—	0.38	0.51	0.38	0.51	10.83	12.36
L. p. yessoensis[b]	37	38	37	28	31	2	3	—	0.13	0	0.13	11.21	12.74
L. p. inexpecta (LPIS)	36	37	36	27	30	3	4	1	—	0.13	0.25	11.08	12.61
L. p. inexpecta (LPIS 3)	37	38	37	28	31	2	3	0	1	—	0.13	11.21	12.74
L. p. inexpecta[b]	38	39	38	29	32	3	4	1	2	1	—	11.34	12.87
B. mansfieldi	93	92	91	94	97	86	85	88	87	88	89	—	9.55
B. thaidina	96	97	96	95	98	98	97	100	99	100	101	75	—

[a] Species codes refer to those in Table 1.

[b] *L. japonica* 1 (LJN, LJH, LJO and LJAh), *L. japonica* 2 (LJM, LJA and LJS), *L. p. puziloi* (LPPK and LPPU), *L. p. coreana* (LPCK and LPCN), *L. p. yessoensis* (LPYA and LPYY) and *L. p. inexpecta* (LPIK and LPISm) showed no intraspecific sequence variations.

ences. The percentage sequence differences between *L. japonica* and the other *Luehdorfia* butterflies ranged from 4.20 (33/785) to 4.97% (39/785). *L. japonica* showed 4.20–4.71% (33–37/785) sequence differences from *L. chinensis* and *L. longicaudata* and 4.59–4.97% (36–39/785) from subspecies of *L. puziloi*. *L. chinensis* and *L. longicaudata*, both inhabiting China, showed sequence differences of 1.15% (9/785) to each other and 3.44–4.08% (27–32/785) from subspecies of *L. puziloi*. Subspecies of *L. puziloi* showed 0–0.51% (0–4/785 bases) sequence differences among themselves.

Molecular phylogeny

Phylogenetic trees of the genus *Luehdorfia* based on the mitochondrial ND5 nucleotide sequences were constructed using the NJ method, the UPGMA method and the MP method (Fig. 3). These trees indicated that species of the *Luehdorfia* butterflies can be divided into three groups. The first group consists of only *L. japonica*. The second group consists of *L. chinensis* and *L. longicaudata*, and the third group consists of subspecies of *L. puziloi*. High bootstrap values supported close relationships of the three groups. The divergence of the subspecies of *L. puziloi* is not certain because the bootstrap values are low (Fig. 3a). Phylogenetic relationships among populations of each species were not detected, probably because of very few phylogenetic information (Fig. 2 and Table 2).

Discussion

Shinkawa (1991) and Watanabe (1996) suggested that *L. puziloi* first colonized the Japanese Archipelago, and that *L. chinensis* diverged first from the ancestral butterflies of the genus *Luehdorfia*. It may be hypothesized that *L. japonica* diverged from *L. p. inexpecta* as a result of host plant alteration from the deciduous genus *Asiasarum* to the evergreen genus *Asarum*, because *L. p. inexpecta* is restricted to the genus *Asiasarum*, while *L. japonica* not only feeds on the genus *Asarum* but is able to grow on *Asiasarum* (Takahashi, 1973; Hiura, 1978).

However, the phylogenetic tree inferred in the present data (Fig. 3) gave different results from those of Takahashi (1973) and Hiura (1978). The present study showed that *L. japonica* diverged first from the ancestral *Luehdorfia*, and that then the ancestral stock successively yielded the *L. chinensis*–*L. longicaudata* group and *L. puziloi* subspecies.

The host plant of *L. puziloi* is restricted to the genus *Asiasarum*. *Asarum asperum* is one of the host plants of *L. japonica* and contains neolignoid which is antifeedant to *L. p. inexpecta* larvae, and the substance is not detectable in the genus *Asiasarum* (Honda *et al.*, 1995). Therefore, there is no possibility that *L. japonica* originated from *L. p. inexpecta* as a result of the colonization of *Asarum* (Takahashi, 1973; Hiura, 1978). The molecular phylogenetic tree based on nucleotide sequences of mitochondrial ND5 (Fig. 3) suggests that *L. japonica* has remained feeding on that

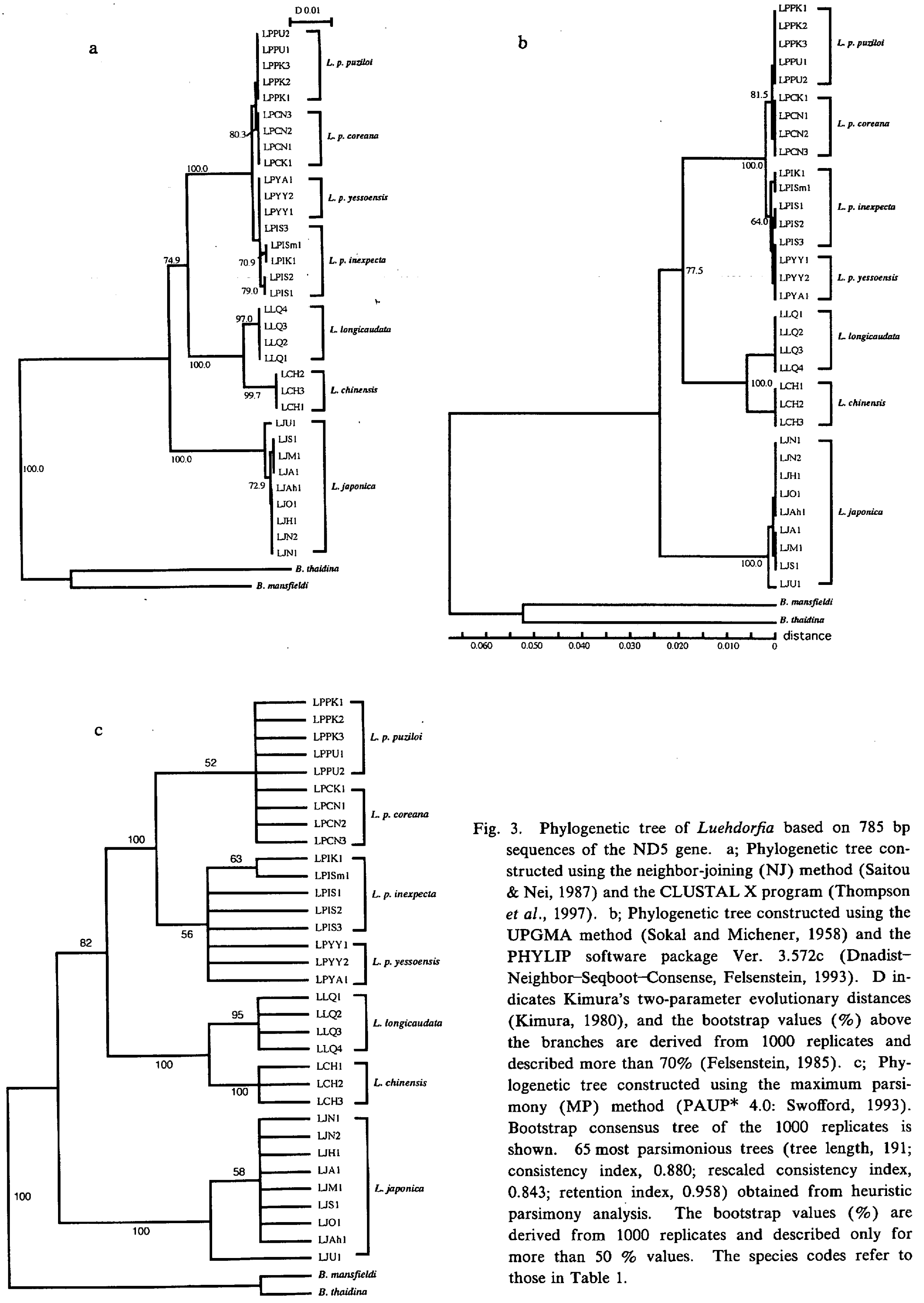

Fig. 3. Phylogenetic tree of *Luehdorfia* based on 785 bp sequences of the ND5 gene. a; Phylogenetic tree constructed using the neighbor-joining (NJ) method (Saitou & Nei, 1987) and the CLUSTAL X program (Thompson *et al.*, 1997). b; Phylogenetic tree constructed using the UPGMA method (Sokal and Michener, 1958) and the PHYLIP software package Ver. 3.572c (Dnadist–Neighbor–Seqboot–Consense, Felsenstein, 1993). D indicates Kimura's two-parameter evolutionary distances (Kimura, 1980), and the bootstrap values (%) above the branches are derived from 1000 replicates and described more than 70% (Felsenstein, 1985). c; Phylogenetic tree constructed using the maximum parsimony (MP) method (PAUP* 4.0: Swofford, 1993). Bootstrap consensus tree of the 1000 replicates is shown. 65 most parsimonious trees (tree length, 191; consistency index, 0.880; rescaled consistency index, 0.843; retention index, 0.958) obtained from heuristic parsimony analysis. The bootstrap values (%) are derived from 1000 replicates and described only for more than 50 % values. The species codes refer to those in Table 1.

host plant which ancestral butterflies fed on.

Branching times were estimated in this study on the basis of substitution rates in mitochondrial DNAs of several arthropods (ca. 1.2% per million years (Myr) per lineage, according to Brower, 1994) and on the basis of the Kimura's D value from the ND5 genes of carabid beetles (the correspondence of the 0.01 D unit with 4±0.5 Myr, according to Osawa *et al.*, 1999). The branching between *L. japonica* and the other *Luehdorfia* butterflies was estimated to have occurred 1.75–2.05 Myr ago using substitution rates in mitochondrial DNAs (as corresponding to 4.20–4.97% sequence differences), and inferred 9.6±1.2 Myr ago using the D value (0.024 in Fig. 3b). The time scale inferred from the Brower's rates, however, may be the minimal ones (Su *et al.*, 1996a). The rate for carabids seems impractical to the *Luehdorfia* butterflies. However, since the carabid rates are based on the mitochondrial ND5 genes, the use of the rate is well justified.

These branching times correspond to the latest part of the Tertiary of the Cenozoic era. An evolutionary scenario is assumed that a certain ancestral butterfly of the genus *Luehdorfia* reached the area in and around the current Japan, and that later *L. japonica* fed on the host plant of the ancestral butterflies. After the branching of *L. japonica*, other ancestral *Luehdorfia* may have extended to northern areas, adapting to *Asiasarum* as host plant and have diverged into two groups, one of which includes *L. chinensis* and *L. longicaudata* and the other of which includes *L. puziloi* subspecies. It may be conceivable that the *L. puziloi* subspecies would diverge at almost the same time, because they are closely related with each other. The phylogeny of *L. puziloi* subspecies, however, is not elucidated in this study.

Acknowledgments

We thank Dr. Hideo Mohri, Director of the National Institute for Basic Biology, for promotion of this study, Drs. Syozo Osawa, Zhi-Hui Su, Takeshi Ohama and other staffs of the Biohistory Research Hall for their valuable advices, and Dr. Noriyuki Nasu, Professor Emeritus, University of Tokyo, and Dr. Takashi Hamada, Professor of The University of the Air, for palaeogeographical advices. We are grateful to Dr. Vladimir Kononenko of Institute of Biology and Pedology, Far East Scientific Center, USSR Academy of Sciences and Japanese amateur entomologists specializing in butterflies, Shigeru Yamaya, Fujio Tsukui, Kouji Muroya, Hiroshi Miura, Shigeaki Moroboshi, Kazumitsu Kitawaki and Mie Fukushima for providing samples. We also express our heartfelt thanks to many amateur butterfly specialists in China, Korea, Russia and Japan for providing samples of various species.

References

Brower, A. V. Z. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proceeding National Academy Science of U.S.A.*, **91**: 6491–6495.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution*, **39**: 783–791.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Hiura, I. 1978. Where from Originate Butterflies: 70–82. Soju Shobo, Tokyo. (In Japanese.)

Honda, K. 1980. Osmeterial secretions of Papilionid larvae in the genera *Luehdorfia, Graphium* and *Atrophaneura* (Lepidoptera). *Insect Biochemistry*, **10**: 583–588.

Honda, K., Saitoh, T. & Hara, S. 1995. A neolignoid feeding against *Luehdorfia puziloi* larvae (Lepidoptera: Papilionidae) from Heterotropa aspera, a host plant of sibling species, *L. japonica. Journal of Chemical Ecology*, **21**: 1541–1548.

Ishizuka, Y. 1980. The study of relationships of the genus *Luehdorfia. Konchu to Shizen*, **15**: 13–17. (In Japanese.)

Ishizuka, Y. 1991. The study of relationships of the genus *Luehdorfia. Konchu to Shizen*, **26**: 21–29. (In Japanese.)

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**: 111–120.

Kominami, H., Yamaguchi, O. & Sumiyoshi, K. 1987. Species diversity in the island populations of *Papilio bianor* complex in Japan considered from gene flow and genetic distance. *Tyo to Ga*, **38**: 269–286.

Kudoh, T. & Inaoka, S. 1995. Interspecific crossing between *Luehdorfia longicaudata* Lee and other species of the genus *Luehdorfia* Crueger (Pap.). *Butterflies*, **11**: 16–11. (In Japanese.)

Osawa, S., Su, Z. H., Kim, C. G., Okamoto, M., Tominaga, O. & Imura, Y. 1999. Evolution of the carabid ground beetles. *Advances in Biophysics*, **36**: 65–106.

Saigusa, T. 1973. On the phylogeny of the genus *Luehdorfia. Konchu to Shizen*, **8**: 5–18. (In Japanese.)

Saiki, R. K., Gelfand, D. H., Stoffel, S., Schaft, S. T., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**: 487–491.

Saitoh, K. 1988. Chromosomes of Butterflies from morphological studies in 1966–1984. *Special bulletin of the Lepidopterological Society of Japan*, **6**: 499–525. (In Japanese.)

Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology of Evolution*, **4**: 406–425.

Sanger, F., Nicklen, S. & Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proceeding National Academy Science of U.S.A.*, **74**: 5463–5467.

Shinkawa, T. 1991. The study of relation between genus *Luehdorfia*. *Konchu to Shizen*, **26**: 11–20. (In Japanese.)

Shinkawa, T. 1996. Where did *Luehdorfia japonica* come from? *Konchu to Shizen*, **31**: 18–21. (In Japanese.)

Sokal, R. R. & Michener, C. D. 1958. A statistical method for evaluating systematic relationship. *University of Kansas Science Bulletin*, **28**: 1409–1438.

Su, Z. H., Ohama, T., Okada, T. S., Nakamura, K., Ishikawa, R. & Osawa, S. 1996a. Phylogenetic relationships and evolution of the Japanese Carabinae ground beetles based on mitochondrial ND5 gene sequences. *Journal of Molecular Evolution*, **42**: 124–129.

Su, Z. H., Ohama, T., Okada, T. S., Nakamura, K., Ishikawa, R. & Osawa, S. 1996b. Geography-linked phylogeny of the *Damaster* ground beetles inferred from mitochondrial ND 5 gene sequences. *Journal of Molecular Evolution*, **42**: 130–134.

Su, Z. H., Tominaga, O., Ohama, T., Kajiwara, E., Ishikawa, R., Okada, T. S., Nakamura, K. & Osawa, S. 1996c. Parallel evolution in radiation of *Ohomopterus* ground beetles inferred from mitochondrial ND5 gene sequences. *Journal of Molecular Evolution*, **43**: 662–671.

Swofford, D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.0. Sinauer Associates, Sunderland, Massachusetts.

Takahashi, A. 1973. The theory of distribution "*Luehdorfia Japonica*". *Konchu to Shizen*, **8**: 2–7. (In Japanese.)

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **24**: 4876–4882.

Watanabe, Y. 1996. Phylogenetic classification. In Watanabe, Y. (ed.), *Monograph of Luehdorfia Butterflies*: 145–150. Hokkaido Univ. Press, Hokkaido. (In Japanese.)

Yagi, T., Sasaki, G. & Takebe, H. 1999. Phylogeny of Japanese papilionid butterflies inferred from nucleotide sequences of the mitochondrial ND5 gene. *Journal of Molecular Evolution*, **48**: 42–48.

(Received October 4, 1999; Accepted February 22, 2000)