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Molecular systematics and biogeography of the genus *Zizina* (Lepidoptera: Lycaenidae)

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Abstract

Butterflies of the genus *Zizina* are widely distributed in all zoogeographical regions except the New World (North and South America) and the northern part of Eurasia. We address some of the problems in regard to the taxonomy and biogeography of the genus. We inferred phylogenetic relationships for all four species in the current classification of this genus from the ND5 region of mtDNA. From our molecular analyses and morphological evidence, we concluded that this genus contains three species; *Z. otis*, *Z. oxleyi* and *Z. emelina*. The status of the latter species is revised, while *Z. labradus* and *Z. antanossa*, which were formerly treated as specifically distinct, are regarded as subspecies of *Z. otis*. Based on our analyses, we also employ phylogeography to discuss possible speciation events in the genus. Each of the three *Zizina* species appears to have branched from the common ancestor, with a divergence time estimated to be about 2.5 million years ago. The ancestors of *Z. oxleyi* and *Z. emelina* are postulated to have adapted to a temperate climate, diverged in the northern and southern hemispheres, and resulted in the extant species from New Zealand and East Asia, respectively. In contrast, the ancestor of *Z. otis* adapted mainly to tropical and subtropical zones, and the extant *Z. otis* dispersed into the Afrotropical, Oriental and Australian regions. Feeding adaptations in the larvae also might have had an effect on speciation within *Zizina*. In addition, our results indicated that there is a possibility that the distribution of the New Zealand *Z. oxleyi* was reduced in extent by the introduction of *Z. otis*, which immigrated recently from Australia or its surroundings.

Key words: classification, conservation, evolution, introduction, lycaenid butterfly, molecular clock, molecular phylogeny, mtDNA, phylogeography, Polyommatini, speciation

Introduction

Members of the lycaenid butterfly genus *Zizina* Chapman occur in tropical to temperate zones of the Palaearctic, Oriental, Australian and Afrotropical regions (Chapman 1910; Corbet & Pendlebury 1956, 1992; Parsons 1999). Although the genus is widely distributed, it includes some species or subspecies that have shown recent range-size decreases. This is because they often occur in environments that are easily degraded, such as natural grasslands, riversides and coastlines.

In appearance, this genus is similar to two allied genera, *Zizeeria* Chapman and *Pseudozizeeria* Beuret, but is characterized by asymmetrical androconia with a concave distal margin, a black postdiscal dot shifted

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inwardly in cell 6 of the ventral hindwing, and one or two quite long subbasal bristles on the ventral margin of the male genital valva (Chapman 1910; Eliot 1973; Corbet & Pendlebury 1992).

According to Bridges (1988), whose classification appears to be widely accepted, *Zizina* presently comprises the following four species: *Z. otis* (Fabricius), *Z. labradus* (Godart), *Z. oxleyi* (C. & R. Felder) and *Z. antanossa* (Mabille). The four species are distinguished from one another by their geographical distribution (Fig. 1) and the features of the wing markings (discussed in detail later). *Zizina otis* shows considerable geographical variation, occurring in the Oriental region and parts of the Palaearctic region (Seki *et al.* 1991; Vane-Wright & de Jong 2003), and also in a narrow area of western Egypt (Larsen 1989). *Zizina labradus*, which is characterized by rows of faint large gray spots on the ventral surface of the wings, is found in the Australian region, including Melanesia and Polynesia (Parsons 1999). Although this species is considered to replace *Z. otis* in this region, the delimitation of the two species is unclear and has not been addressed in the literature. *Zizina oxleyi*, a New Zealand endemic, has a dark ground color and brown markings along the outsides of the postdiscal black spots on the wing underside. *Zizina antanossa* is widely distributed in the Afrotropical region and is characterized by scattered pale blue scales on the male dorsal wing surface.

The classification of the genus by Bridges (1988) is not stable. According to Corbet and Pendlebury (1992), because the male genitalia of *Zizina* exhibit slight but constant differences in each region, *Z. otis indica* (Murray) from Peninsular India and Ceylon is included in the African *Z. antanossa*. Furthermore, *Z. oxleyi* is synonymized with *Z. labradus*. Gibbs (1980) treated *Z. oxleyi* as a subspecies of *Z. labradus* or *Z. otis*. Chapman (1910) and Stempffer (1967) classified the genus into three species: *Z. antanossa* from Africa, *Z. indica* from India and *Z. labradus* from the Oriental and Australian regions. Parsons (1999) treated all *Zizina* populations excluding *Z. antanossa* as *Z. labradus*. D'Abrera (1978, 1980, 1986) recognized two or three species: *Z. otis* from the Oriental and Australian regions, *Z. antanossa* from Africa and a different species from India [*Z. antanossa*?] which is distinguishable from *Z. otis*. Corbet (1948) regarded all *Zizina* groups as only *Z. otis*. Thus *Zizina* is regarded by different authors as having from one to four species.

The reasons for this taxonomic uncertainty, even though the geographical distribution has been well investigated, are as follows: 1) morphological differences of the male genitalia, which are frequently important as taxonomic characters, are not helpful in the genus, 2) geographical variation of the wing markings do not correspond with those of the male genitalia, 3) the geographical borders between neighboring species are unclear, 4) hybrid-like individuals are occasionally observed in overlapping areas of the ranges of two species or subspecies, and 5) there has been no detailed worldwide work on the specific classification of the genus. It would be of interest to examine whether or not populations are capable of hybridization.

In *Zizina*, a genus that is difficult to classify on morphology alone, one effective method to assess the possibility of present or recent gene flow among populations is through phylogenetic studies using mtDNA. On the other hand, early workers have provided some fascinating discussions concerning evolutionary processes and the biogeography of *Zizina* because this genus occurs also on ocean islands and in areas where artificial introduction is suspected to have taken place (Stempffer 1967; Kawazoé & Wakabayashi 1976; Gibbs 1980; Larsen 1989). Thus, it is of much interest to elucidate the phylogeography of this genus.

In this paper we propose a sound classification of *Zizina* in accordance with the molecular phylogeny inferred from mtDNA and the external morphology based on male wing markings and genitalia. Additionally, we discuss the phylogeny, evolution and biogeography of the genus.

Materials and Methods

1. Material and morphology

All four currently recognized *Zizina* species, *Z. otis*, *Z. labradus*, *Z. oxleyi* and *Z. antanossa*, were studied from a total of 181 adults (Fig. 1), of which scientific names and detailed data are listed in Table 1. The subspecific names of only *Z. otis* are listed, in accordance with recent studies (Seki *et al*. 1991; Vane-Wright & de Jong 2003; Yago 2007). Those from Indonesia are not ranked but represented as "ssp.", following Vane-Wright and de Jong (2003). Although Seki *et al*. (1991) recognized all *Z. otis* from SE Asia as the nominotypical subspecies, we avoid this treatment because the Indonesian *Z. otis* appears to have some geographical variation on the wing underside. As out-group taxa, both species of *Zizeeria* [*Z. karsandra* (Moore) and *Z. knysna* (Trimen)] and the single species of *Pseudozizeeria* [*P. maha* (Kollar)] were used (Table 1). The two genera, together with *Zizina*, comprise the *Zizeeria* section of the tribe Polyommatini *sensu* Eliot (1973). Additionally, one species of *Scolitantides* Hübner [*S. orion* (Pallas)], relatively closely allied to *Zizina*, was used.

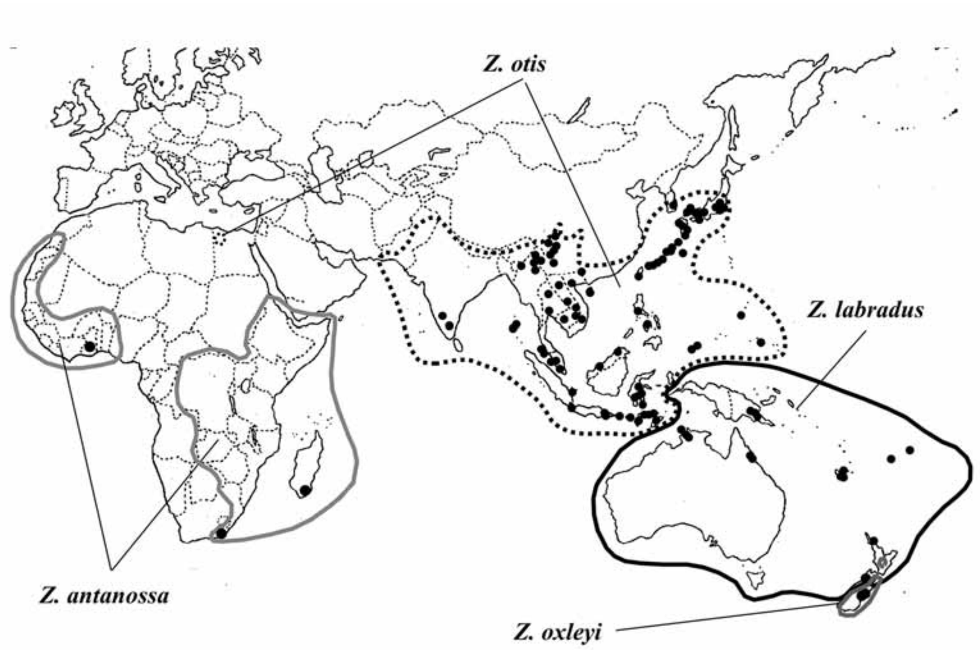

FIGURE 1. Geographical distribution of all four currently recognized species of *Zizina*. The specific classification follows Bridges (1988). Dots indicate the localities of the 181 samples used in this study. As for distribution, we cited the data of the collection in The Natural History Museum and other literatures (Ackery *et al*. 1996; Bascombe *et al*. 1999; Braby 2004; Chapman 1910; Chou 1994; Clark & Dickson 1971; Common & Waterhouse 1981; Corbet 1948; Corbet & Pendlebury 1956, 1992; D'Abrera 1978, 1980, 1986; Duke *et al*. 1999; Ek-Amnuay 2006; Evans 1932; Fermon *et al.* 2001; Fox *et al.* 1965; Fukuda *et al*. 1986; Gibbs 1980; Khatri 2004; Kielland 1990; Kim 2002; Kinyon 2003; Koiwaya 1989; Larsen 1984, 1989, 1996, 2005; Leech 1892-1894; Monastyrskii & Devyatkin 2003; Osada 1999; Parsons 1999; Pinratana 1981; Pringle *et al.* 1994; Seitz 1927; Seki *et al*. 1991; Shirôzu 1960; Smith 1989; Stempffer 1967; Takanami 1986; Tennent 2002; Vane-Wright & de Jong 2003; Wang & Fan 2002; Williams 1989).

For the examination of male genitalia, apical parts of the abdomen were placed in 5 % KOH solution at about 50 ℃ for 3-5 hours, then neutralized in 2 % acetic acid. After this treatment, they were washed with distilled water and placed in 80 % ethanol for dissection and examination. Chlorazol black E was used to stain the KOH-treated integument. The genitalia were examined and illustrated using Olympus SZ60 and Leica MZ8 stereoscopic microscopes with magnifications of up to × 189. Terminology of the male genitalia followed Shirôzu (1960), except for the substitution of 'falx' for 'brachium'. In describing wing markings we largely adopted the system proposed by Schwanwitsch (1949).

TABLE 1. Samples used in this study. The specific classification followed Bridges (1988), and the subspecific classification of *Z. otis*, follows mainly Seki *et al*. (1991), Vane-Wright (2003) and Yago (2007) (See also Materials and Methods in text). Second column indicates abbreviated scientific names and localities. *ZO*: *Zizina otis otis* or *Zizina otis* ssp., *ZOr*: *Zizina otis riukuensis, ZOe*: *Zizina otis emelina, ZOt*: *Zizina otis thibetensis*, *ZOi*: *Zizina otis indica*, *ZA*: *Zizina antanossa*, *ZL*: *Zizina labradus*, *ZOX*: *Zizina oxleyi.*

Taxon	Sample name	Sex	Locality	GenBank Accession No.	GC Content (%)
In group					
Zizina otis emelina	*ZOe*-Korea1	Male	Yeonghae-myeon, Yeongdeok-gun, Gyeongsangbuk-do, South Korea	AB359470	16.9
Zizina otis emelina	*ZOe*-Korea2	Female	Yeonghae-myeon, Yeongdeok-gun, Gyeongsangbuk-do, South Korea	AB359471	16.9
Zizina otis emelina	*ZOe*-Japan1	Male	Nishi, Kamogawa City, Chiba Pref., Japan	AB359472	16.6
Zizina otis emelina	*ZOe*-Japan2	Female	Nishi, Kamogawa City, Chiba Pref., Japan	AB359473	16.6
Zizina otis emelina	*ZOe*-Japan3	Female	Nishi, Kamogawa City, Chiba Pref., Japan	AB359474	16.6
Zizina otis emelina	*ZOe*-Japan4	Male	Nishi-konishi, Kamogawa City, Chiba Pref., Japan	AB359475	16.6
Zizina otis emelina	*ZOe*-Japan5	Female	Iriuda, Maruyama-machi, Awa-gun, Chiba Pref., Japan	AB359476	16.6
Zizina otis emelina	*ZOe*-Japan6	Male	Iriuda, Maruyama-machi, Awa-gun, Chiba Pref., Japan	AB359477	16.6
Zizina otis emelina	*ZOe*-Japan7	Female	Mukohgawara, Ujiie-cho, Shioya-gun, Tochigi Pref., Japan	AB359478	16.7
Zizina otis emelina	*ZOe*-Japan8	Male	Asahara, Wakakusa-cho, Nakakomo-gun, Yamanashi Pref., Japan	AB359479	16.7
Zizina otis emelina	*ZOe*-Japan9	Female	Asahara, Minami-Alps City, Yamanashi Pref., Japan	AB359480	16.7
Zizina otis emelina	*ZOe*-Japan10	Male	Asahara, Minami-Alps City, Yamanashi Pref., Japan	AB359481	16.7
Zizina otis emelina	*ZOe*-Japan11	Male	Asahara, Minami-Alps City, Yamanashi Pref., Japan	AB359482	16.7
Zizina otis emelina	*ZOe*-Japan12	Male	Asahara, Minami-Alps City, Yamanashi Pref., Japan	AB359483	16.7
Zizina otis emelina	*ZOe*-Japan13	Male	Asahara, Minami-Alps City, Yamanashi Pref., Japan	AB359484	16.7
Zizina otis emelina	*ZOe*-Japan14	Male	Shimoimasuwa, Minami-Alps City, Yamanashi Pref., Japan	AB359485	16.7
Zizina otis emelina	*ZOe*-Japan15	Male	Tatomi-cho, Nakakoma-gun, Yamanashi Pref., Japan	AB359486	16.7
Zizina otis emelina	*ZOe*-Japan16	Male	Nagai, Yokosuka City, Kanagawa Pref., Japan	AB359487	16.7
Zizina otis emelina	*ZOe*-Japan17	Female	Nagai, Yokosuka City, Kanagawa Pref., Japan	AB359488	16.7
Zizina otis emelina	*ZOe*-Japan18	Male	Isawada, Awa-cho, Awa-gun, Tokushima Pref., Japan	AB359489	16.7
Zizina otis emelina	*ZOe*-Japan19	Male	Hashirii, Toyonaka City, Osaka Pref., Japan	AB359490	16.9
Zizina otis emelina	*ZOe*-Japan20	Male	Hashirii, Toyonaka City, Osaka Pref., Japan	AB359491	16.7
Zizina otis emelina	*ZOe*-Japan21	Male	Hashirii, Toyonaka City, Osaka Pref., Japan	AB359492	16.7
Zizina otis emelina	*ZOe*-Japan22	Male	Shimogawara, Itami City, Hyogo Pref., Japan	AB359493	16.9
Zizina otis emelina	*ZOe*-Japan23	Male	Takada-cho, Ono City, Hyogo Pref., Japan	AB359494	16.7
Zizina otis emelina	*ZOe*-Japan24	Male	Kotaka, Takino-cho, Kato-gun, Hyogo Pref., Japan	AB359495	16.7
Zizina otis emelina	*ZOe*-Japan25	Male	Higashi-tokuhisa, Nanko-cho, Sayo-gun, Hyogo Pref., Japan	AB359496	16.7
Zizina otis emelina	*ZOe*-Japan26	Male	Shimodoi, Wakasano-cho, Aioi City, Hyogo Pref., Japan	AB359497	16.7
Zizina otis emelina	*ZOe*-Japan27	Male	Nishiune, Ako City, Hyogo Pref., Japan	AB359498	16.7
Zizina otis emelina	*ZOe*-Japan28	Male	Midori-cho (Awaji Is.), Mihara-gun, Hyogo Pref., Japan	AB359499	16.7
Zizina otis emelina	*ZOe*-Japan29	Male	Ikutabata, Hokudan-cho (Awaji Is.), Tsuna-gun, Hyogo Pref., Japan	AB359500	16.7

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TABLE 1 (continued)

Taxon	Sample name	Sex	Locality	GenBank Accession No.	GC Content (%)
In group					
Zizina otis emelina	*ZOe*-Japan30	Male	Hirafuku, Mimasaka-cho, Aida-gun, Okayama Pref., Japan	AB359501	16.7
Zizina otis emelina	*ZOe*-Japan31	Male	Kasuga, Kagamino-cho, Tomata-gun, Okayama Pref., Japan	AB359502	16.7
Zizina otis emelina	*ZOe*-Japan32	Female	Ijirino, Soja City, Okayama Pref., Japan	AB359503	16.7
Zizina otis emelina	*ZOe*-Japan33	Male	Otono, Houki-cho, Saihaku-gun, Tottori Pref., Japan	AB359504	16.7
Zizina otis emelina	*ZOe*-Japan34	Female	Otono, Houki-cho, Saihaku-gun, Tottori Pref., Japan	AB359505	16.7
Zizina otis emelina	*ZOe*-Japan35	Male	Fujiyoshi, Hiezu-son, Saihaku-gun, Tottori Pref., Japan	AB359506	16.7
Zizina otis emelina	*ZOe*-Japan36	Male	Katsura Is., Kaga, Shimane-cho, Matsue City, Shimane Pref., Japan	AB359507	16.7
Zizina otis emelina	*ZOe*-Japan37	Male	Nagoya, Chinsei-cho, Higashi-matsuura-gun, Saga Pref., Japan	AB359508	16.7
Zizina otis emelina	*ZOe*-Japan38	Male	Nabenodaira, Takamori-machi, Aso-gun, Kumamoto Pref., Japan	AB359509	16.7
Zizina otis emelina	*ZOe*-Japan39	Female	Nabenodaira, Takamori-machi, Aso-gun, Kumamoto Pref., Japan	AB359510	16.7
Zizina otis emelina	*ZOe*-Japan40	Male	Ikitsuki-cho (Ikitsuki Is.), Kitamatsuura-gun, Nagasaki Pref., Japan	AB359511	16.7
Zizina otis emelina	*ZOe*-Japan41	Male	Tano-cho (200 m), Miyazaki City, Miyazaki Pref., Japan	AB359512	16.7
Zizina otis emelina	*ZOe*-Japan42	Male	Kimotsuki river, Kimotsuki-cho, Kimotsuki-gun, Kagoshima Pref., Japan	AB359513	16.6
Zizina otis emelina	*ZOe*-Japan43	Female	Kimotsuki river, Kimotsuki-cho, Kimotsuki-gun, Kagoshima Pref., Japan	AB359514	16.6
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus1	Male	Takara Is., Toshima-mura, Kagoshima-gun, Kagoshima Pref., Japan	AB359515	17.5
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus2	Male	Takara Is., Toshima-mura, Kagoshima-gun, Kagoshima Pref., Japan	AB359516	17.5
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus3	Male	Kasari-cho (Amami-Ohshima Is.), Oshima-gun, Kagoshima Pref., Japan	AB359517	17.5
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus4	Female	Kasari-cho (Amami-Ohshima Is.), Oshima-gun, Kagoshima Pref., Japan	AB359518	17.5
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus5	Male	Sumiyo-son (Amami-Oshima Is.), Oshima-gun, Kagoshima Pref., Japan	AB359519	17.5
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus6	Male	Sumiyo-son (Amami-Oshima Is.), Oshima-gun, Kagoshima Pref., Japan	AB359520	17.5
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus7	Female	Setouchi-cho (Yoro Is.), Oshima-gun, Kagoshima Pref., Japan	AB359521	17.5
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus8	Male	Kametsu, Tokunoshima-cho (Tokunoshima Is.), Kagoshima Pref., Japan	AB359522	17.5
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus9	Male	Wadomari-cho (Okinoerabu Is.), Oshima-gun, Kagoshima Pref., Japan	AB359523	17.5
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus10	Male	Kaiho-cho, Okinawa City, Okinawa Pref., Japan	AB359524	17.5
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus11	Male	Minamidaito-son (Minamidaito Is.), Shimajiri-gun, Okinawa Pref., Japan	AB359525	17.2
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus12	Female	Kuninaka, Irabu-cho (Shimoji Is.), Miyako-gun, Okinawa Pref., Japan	AB359526	17.4
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus13	Male	Kuninaka, Irabu-cho (Shimoji Is.), Miyako-gun, Okinawa Pref., Japan	AB359527	17.4
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus14	Male	Ishigaki Bay, Ishigaki City (Ishigaki Is.), Okinawa Pref., Japan	AB359528	17.4
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus15	Female	Fuzaki, Ishigaki City (Ishigaki Is.), Okinawa Pref., Japan	AB359529	17.4
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus16	Male	Nakasuji, Taketomi-cho (Taketomi Is.), Yaeyama-gun, Okinawa Pref., Japan	AB359530	17.4
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus17	Male	Taketomi-cho (Kuro Is.), Yaeyama-gun, Okinawa Pref., Japan	AB359531	17.4
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus18	Female	Taketomi-cho (Kuro Is.), Yaeyama-gun, Okinawa Pref., Japan	AB359532	17.4
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus19	Male	Komi, Taketomi-cho (Iriomote Is.), Yaeyama-gun, Okinawa Pref., Japan	AB359533	17.4

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TABLE 1 (continued)

Taxon	Sample name	Sex	Locality	GenBank Accession No.	GC Content (%)
In group					
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus20	Female	Hikawa, Yonaguni-cho (Yonaguni Is.), Yaeyama-gun, Okinawa Pref., Japan	AB359534	17.4
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus21	Male	Hikawa, Yonaguni-cho (Yonaguni Is.), Yaeyama-gun, Okinawa Pref., Japan	AB359535	17.4
Zizina otis riukuensis	*ZOr*-Japan-Ryukyus22	Female	Hikawa, Yonaguni-cho (Yonaguni Is.), Yaeyama-gun, Okinawa Pref., Japan	AB359536	17.4
Zizina otis riukuensis	*ZOr*-Taiwan	Male	Eluanbi, Hengchun Town, Pingtung County, Taiwan	AB359537	17.4
Zizina otis thibetensis	*ZOt*-China-Sichuan1	Female	Qiongla Shan, Wenchuan, Sichuan, China	AB359538	16.6
Zizina otis thibetensis	*ZOt*-China-Sichuan2	Male	Emei Shan, Leshan City, Sichuan, China	AB359539	16.6
Zizina otis thibetensis	*ZOt*-China-Yunnan1	Male	Xi Shan, Kunming City, Yunnan, China	AB359540	16.6
Zizina otis thibetensis	*ZOt*-China-Yunnan2	Male	Guangmao Shan, Zhanhe (near Yongsheng), Yunnan, China	AB359541	16.6
Zizina otis thibetensis	*ZOt*-China-Yunnan3	Female	Guangmao Shan, Zhanhe (near Yongsheng), Yunnan, China	AB359542	16.6
Zizina otis thibetensis	*ZOt*-China-Guangxi	Male	Jingxi County, Guangxi, China	AB359543	16.6
Zizina otis otis	*ZO*-China-Sichuan	Male	Dayi County, Sichuan, China	AB359544	17.4
Zizina otis otis	*ZO*-China-Guangxi	Male	Jingxi County, Guangxi, China	AB359545	17.1
Zizina otis otis	*ZO*-China-Hainan	Male	Diaoluoshang, Hainan Is., China	AB359546	17.4
Zizina otis otis	*ZO*-Vietnam1	Male	Bai Tu Long, Ba Mun Is., Quang Ninh Prov., Vietnam	AB359547	17.5
Zizina otis otis	*ZO*-Vietnam2	Female	Dambri (850 m), Bao Loc, Vietnam	AB359548	17.4
Zizina otis otis	*ZO*-Vietnam3	Male	Da Nang City, Vietnam	AB359549	17.4
Zizina otis otis	*ZO*-Laos1	Female	Vangviang, Vientiane, Laos	AB359550	17.4
Zizina otis otis	*ZO*-Laos2	Male	Xiangngeun, Luang Phabang, Laos	AB359551	17.4
Zizina otis otis	*ZO*-Laos3	Male	Pakxan, Bolikhamxai, Laos	AB359552	17.4
Zizina otis otis	*ZO*-Laos4	Male	Pakxan, Bolikhamxai, Laos	AB359553	17.4
Zizina otis otis	*ZO*-Laos5	Female	Lak Sao, Bolikhamxai, Laos	AB359554	17.4
Zizina otis otis	*ZO*-Laos6	Male	Lak Sao, Bolikhamxai, Laos	AB359555	17.4
Zizina otis otis	*ZO*-Laos7	Male	Attapeu, Attapeu, Laos	AB359556	17.3
Zizina otis otis	*ZO*-Cambodia1	Male	Poy, Ratanakiri, Cambodia	AB359557	17.4
Zizina otis otis	*ZO*-Cambodia2	Male	Pramaoy, Pouthisat, Cambodia	AB359558	17.4
Zizina otis otis	*ZO*-Cambodia3	Male	Pramaoy, Pouthisat, Cambodia	AB359559	17.4
Zizina otis otis	*ZO*-Thailand1	Female	Cham Pasak, Chiang Mai, Thailand	AB359560	17.3
Zizina otis otis	*ZO*-Thailand2	Male	Bangkok, Thailand	AB369064	17.4
Zizina otis otis	*ZO*-Thailand3	Male	Bangkok, Thailand	AB369065	17.4
Zizina otis otis	*ZO*-Thailand4	Male	Kathu, Phuket Is., Thailand	AB359561	17.4
Zizina otis thibetensis	*ZOt*-Myanmar1	Male	Panwa (2,000 m), Kachin State, Myanmar	AB359562	17.4
Zizina otis thibetensis	*ZOt*-Myanmar2	Male	Panwa (2,000 m), Kachin State, Myanmar	AB359563	17.4
Zizina otis otis	*ZO*-Myanmar1	Male	Namti (300 m), West Myitkyina, Kachin State, Myanmar	AB359564	17.4

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TABLE 1 (continued)

Taxon	Sample name	Sex	Locality	GenBank Accession No.	GC Content (%)
In group					
Zizina otis otis	*ZO*-Myanmar2	Male	Myitkyina, Kachin State, Myanmar	AB359565	17.4
Zizina otis otis	*ZO*-Myanmar3	Female	Kalalu (140 m), Shan State, Myanmar	AB359566	17.4
Zizina otis otis	*ZO*-Myanmar4	Male	Nanyun (600 m), Sagaing State, Myanmar	AB359567	17.4
Zizina otis indica	*ZOi*-India1	Male	Mysore, Karnataka State, India	AB359568	17.1
Zizina otis indica	*ZOi*-India2	Male	Mysore, Karnataka State, India	AB359569	17.1
Zizina otis indica	*ZOi*-India3	Male	Mysore, Karnataka State, India	AB359570	17.1
Zizina otis indica	*ZOi*-India4	Male	Coimbatore, Tamil Nadu State, India	AB359571	17.1
Zizina otis indica	*ZOi*-India5	Female	Coimbatore, Tamil Nadu State, India	AB359572	17.1
Zizina otis indica	*ZOi*-India6	Female	Coimbatore, Tamil Nadu State, India	AB359573	17.1
Zizina otis otis	*ZO*-Andaman1	Male	Neil Is., South Andaman Iss., India	AB359574	17.1
Zizina otis otis	*ZO*-Andaman2	Male	Havelock Is., South Andaman Iss., India	AB359575	17.1
Zizina otis otis	*ZO*-Langkawi1	Male	Bunting Is., Langkawi, Malaysia	AB359576	17.4
Zizina otis otis	*ZO*-Langkawi2	Male	Bunting Is., Langkawi, Malaysia	AB359577	17.4
Zizina otis otis	*ZO*-Langkawi3	Male	Bunting Is., Langkawi, Malaysia	AB359578	17.4
Zizina otis otis	*ZO*-Penang	Male	Penang Is., Penang State, Malaysia	AB359579	17.4
Zizina otis otis	*ZO*-Malaysia1	Male	Cameron Highlands, Pahang State, Malaysia	AB359580	17.4
Zizina otis otis	*ZO*-Malaysia2	Male	Central Malacca, Malacca State, Malaysia	AB367129	17.4
Zizina otis otis	*ZO*-Borneo1	Female	Tabin, Sabah State, Borneo, Malaysia	AB359581	17.4
Zizina otis otis	*ZO*-Borneo2	Female	Lambir, Sarawak State, Borneo, Malaysia	AB359582	17.4
Zizina otis otis	*ZO*-Phillipines1	Female	Tagaytay, Luzon Is., Philippines	AB359583	17.4
Zizina otis otis	*ZO*-Phillipines2	Male	Dumaguete City, Negros Is., Philippines	AB359584	17.2
Zizina otis otis	*ZO*-Phillipines3	Male	Dumaguete City, Negros Is., Philippines	AB359585	17.2
Zizina otis otis	*ZO*-Guam1	Male	Tumon, Guam, USA	AB359586	17.4
Zizina otis otis	*ZO*-Guam2	Male	Tumon, Guam, USA	AB359587	17.4
Zizina otis otis	*ZO*-Palau1	Male	Angaur Is., Palau	AB359588	17.4
Zizina otis otis	*ZO*-Palau2	Male	Pelillu Is., Palau	AB359589	17.4
Zizina otis otis	*ZO*-Micronesia1	Male	Moen Is., Chuuk State, Micronesia	AB369720	17.4
Zizina otis otis	*ZO*-Micronesia2	Male	Moen Is., Chuuk State, Micronesia	AB369721	17.4
Zizina otis otis	*ZO*-Micronesia3	Male	Moen Is., Chuuk State, Micronesia	AB369722	17.4
Zizina otis otis	*ZO*-Bangka	Male	Bangka Is. (near Sumatra), Indonesia	AB359590	17.3
Zizina otis ssp.	*ZO*-Kurakatau	Female	Anak Krakatau (0 m), Krakarau Is., Indonesia	AB359591	17.3
Zizina otis ssp.	*ZO*-Java1	Male	Secuti, Java, Indonesia	AB359592	17.3
Zizina otis ssp.	*ZO*-Java2	Male	Near Mt. Ar Juno (800 m), Tretes, Java, Indonesia	AB359593	17.3

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TABLE 1 (continued)

Taxon	Sample name	Sex	Locality	GenBank Accession No.	GC Content (%)
In group					
Zizina otis ssp.	*ZO*-Bali1	Male	Uluwatu, Bali, Indonesia	AB359594	17.2
Zizina otis ssp.	*ZO*-Bali2	Female	Uluwatu, Bali, Indonesia	AB359595	17.3
Zizina otis ssp.	*ZO*-Lombok1	Male	Senggigi, Lombok, Indonesia	AB359596	17.3
Zizina otis ssp.	*ZO*-Lombok2	Male	Senggigi, Lombok, Indonesia	AB359597	17.2
Zizina otis ssp.	*ZO*-Flores1	Female	Wengor, Sikka, Flores, Indonesia	AB359598	17.2
Zizina otis ssp.	*ZO*-Flores2	Male	Wengor, Sikka, Flores, Indonesia	AB359599	17.2
Zizina otis ssp.	*ZO*-Flores3	Male	Wengor, Sikka, Flores, Indonesia	AB359600	17.2
Zizina otis ssp.	*ZO*-Flores4	Female	Kimanbuleng, Flores, Indonesia	AB359601	17.2
Zizina otis ssp.	*ZO*-Adonara1	Male	Adonara Is. (near Flores), Indonesia	AB359602	17.2
Zizina otis ssp.	*ZO*-Adonara2	Male	Adonara Is. (near Flores), Indonesia	AB359603	17.1
Zizina otis ssp.	*ZO*-Timor1	Male	Lalendo, Kupang, Timor, Indonesia	AB359604	17.2
Zizina otis ssp.	*ZO*-Timor2	Male	Bau Mata, Kupang, Timor, Indonesia	AB359605	17.2
Zizina otis ssp.	*ZO*-Sulawesi1	Male	Masamba (50-100 m), Central Sulawesi, Indonesia	AB359606	17.3
Zizina otis ssp.	*ZO*-Sulawesi2	Male	Masamba (50-100 m), Central Sulawesi, Indonesia	AB359607	17.3
Zizina otis ssp.	*ZO*-Sulawesi3	Male	Bengobengo (520 m), South Sulawesi, Indonesia	AB359608	17.3
Zizina otis ssp.	*ZO*-Sulawesi4	Male	Kesei (500 m), South Sulawesi, Indonesia	AB359609	17.3
Zizina otis ssp.	*ZO*-Kabaena	Male	Kabaena Is. (near Sulawesi), Indonesia	AB359610	17.2
Zizina antanossa	*ZA*-Ghana	Male	Aburi, Ghana	AB359611	17.2
Zizina antanossa	*ZA*-S.Africa1	Male	Cintsa West, Eastern Cape Prov., South Africa	AB359612	17.2
Zizina antanossa	*ZA*-S.Africa2	Female	Cintsa West, Eastern Cape Prov., South Africa	AB359613	17.2
Zizina antanossa	*ZA*-Madagascar	Male	Tolagnaro, Madagascar	AB367130	17.2
Zizina labradus	*ZL*-NewGuinea1	Male	Okapa, Eastern Highlands Prov., Papua New Guinea	AB359614	17.5
Zizina labradus	*ZL*-NewGuinea2	Male	Wau, Morobe Prov., Papua New Guinea	AB359615	17.5
Zizina labradus	*ZL*-NewGuinea3	Male	Bulolo, Morobe Prov., Papua New Guinea	AB359616	17.5
Zizina labradus	*ZL*-NewGuinea4	Male	Bulolo, Morobe Prov., Papua New Guinea	AB359617	17.5
Zizina labradus	*ZL*-NewCaledonia1	Male	Ouvea Is., New Caedonia	AB359618	17.4
Zizina labradus	*ZL*-NewCaledonia2	Male	Lifou Is., New Caedonia	AB359619	17.4
Zizina labradus	*ZL*-NewCaledonia3	Male	Mt. Koghi (500 m), Grande Terre, New Caledonia	AB367131	17.4
Zizina labradus	*ZL*-Fiji	Male	Nadi (5-10 m), Viti Levu, Fiji	AB359620	17.4
Zizina labradus	*ZL*-Samoa	Male	Tutuila Is., American Samoa	AB359621	17.4
Zizina labradus	*ZL*-Australia1	Male	Garden Point, Melville Is., Northern Territory, Australia	AB359622	17.3
Zizina labradus	*ZL*-Australia2	Female	Garden Point, Melville Is., Northern Territory, Australia	AB359623	17.3
Zizina labradus	*ZL*-Australia3	Male	Cullen Bay, Darwin, Northern Territory, Australia	AB359624	17.1

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TABLE 1 (continued)

Taxon	Sample name	Sex	Locality	GenBank Accession No.	GC Content (%)
In group					
Zizina labradus	*ZL*-Australia4	Male	Cullen Bay, Darwin, Northern Territory, Australia	AB359625	17.2
Zizina labradus	*ZL*-Australia5	Male	Cullen Bay, Darwin, Northern Territory, Australia	AB367132	17.2
Zizina labradus	*ZL*-Australia6	Male	Leanyer, Darwin, Northern Territory, Australia	AB367133	17.2
Zizina labradus	*ZL*-Australia7	Male	Leanyer, Darwin, Northern Territory, Australia	AB367134	17.2
Zizina labradus	*ZL*-Australia8	Male	Cairns, Queensland, Australia	AB367135	17.3
Zizina labradus	*ZL*-Australia9	Male	Cairns, Queensland, Australia	AB367136	17.3
Zizina labradus	*ZL*-Australia10	Male	Cairns, Queensland, Australia	AB367137	17.4
Zizina labradus	*ZL*-Australia11	Male	Cairns, Queensland, Australia	AB359626	17.3
Zizina labradus	*ZL*-NewZealand1	Male	Oakura, Northland, New Zealand	AB359627	17.1
Zizina labradus	*ZL*-NewZealand2	Female	Oakura, Northland, New Zealand	AB359628	17.1
Zizina labradus	*ZL*-NewZealand3	Female	Charming Creek Walk Way, Ngakawau, West Coast, New Zealand	AB359629	17.1
Zizina oxleyi	*ZOX*-NewZealand1	Male	15 km South from Hanmer Springs, North Canterbury, New Zealand	AB359630	18.2
Zizina oxleyi	*ZOX*-NewZealand2	Male	15 km South from Hanmer Springs, North Canterbury, New Zealand	AB359631	18.0
Zizina oxleyi	*ZOX*-NewZealand3	Male	15 km South from Hanmer Springs, North Canterbury, New Zealand	AB359632	18.0
Zizina oxleyi	*ZOX*-NewZealand4	Male	15 km South from Hanmer Springs, North Canterbury, New Zealand	AB359633	18.1
Zizina oxleyi	*ZOX*-NewZealand5	Female	15 km South from Hanmer Springs, North Canterbury, New Zealand	AB359634	18.1
Zizina oxleyi	*ZOX*-NewZealand6	Female	15 km South from Hanmer Springs, North Canterbury, New Zealand	AB359635	18.1
Zizina oxleyi	*ZOX*-NewZealand7	Female	15 km South from Hanmer Springs, North Canterbury, New Zealand	AB359636	18.2
Out-groups					
Scolitantides orion	*S. orion*	Male	Kiyose, Hidaka-cho, Saru-gun, Hokkaido, Japan	AB359637	17.0
Zizeeria karsandra	*Z. karsandra*	Male	Nishihara, Hirara City (Miyako Is.), Okinawa Pref., Japan	AB359638	17.0
Zizeeria knysna	*Z. knysna*	Male	Foot of Komsberge Pass, Northern Cape Prov., South Africa	AB359639	16.3
Pseudozizeeria maha	*P. maha*	Male	Tatsuiwa, Tsukushino City, Fukuoka Pref., Japan	AB359640	16.2

2. Phylogenetic analyses

DNA of most samples was extracted from mid and hind legs of dried adults collected within the last 20 years. Some extractions were from specimens kept in 99 % ethanol immediately after they had been killed. The samples were dissected, digested in 350 μl of a solution containing 10 mM Tris-HCl (pH 8), 100 mM EDTA, 0.5 % SDS, and 0.5 mg/ml Proteinase K (Takara), at 60 ℃ for 1.5 h. Thereafter, the homogenates were extracted twice with phenol/chloroform. The DNA was precipitated with ethanol, washed with 70 % ethanol, and dissolved in 50 μl of TE buffer (pH 8). Most of the DNA samples used in this study are preserved in the Laboratory of Evolutionary Systematics, Department of Biological Sciences, The University of Tokyo. Some are in the Entomological Laboratory, Graduate School of Life and Environmental Sciences, Osaka Prefecture University.

A part of the mitochondrial ND5 gene was amplified by polymerase chain reaction (PCR) using primers V1, 5'-CCTGTTTCTGCTTTAGTTCA-3' (Yagi *et al.* 1999) and KA1L, 5'-GTTCTAATATAAGGTATAAAT-CATAT-3' (Saigusa *et al.* 2001). Additionally, internal primers LY1F, 5'-GCWGGWATTTCWGCTAAT-TATGA-3', LY2F, 5'-ACWCATGCTATATTTAARGCWTTA-3', LY3F, 5'-GTGGWATTCCTTTTTAKCTGG-3', LY4F, 5'-ACWGGWATAACWATATTTTATAC-3', LY2R, 5'-TAAWGCYTTAAATATAGCATGWGT-3' and LY5R, 5'-GATAWATAAATYATATAAGGATA-3' were used for amplification (Fig. 2). The reactions were carried out in a 10 μl final volume reaction containing Ampdirect® Plus buffer (Shimadzu), 0.5 μM each primer, 1 μl of DNA solution as template and 0.25 units of NovaTaq™ Hot Start DNA polymerase (Shimadzu) using PCR Thermal Cycler MP (Takara) with the following cycling condition: 40 to 45 cycles of denaturation at 94 ℃ for 30 s, annealing at 45 ℃ for 30 s and extension at 72 ℃ for 2 min. The PCR products were treated with shrimp alkaline phosphatase and Exonuclease I, and then directly sequenced using the above primers and the ABI Prism BigDye Terminator v.3.1 cycle sequencing kit on an ABI 3100 automated sequencer.

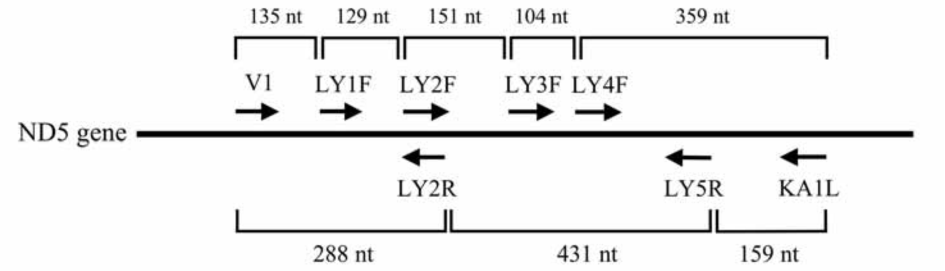


FIGURE 2. A schematic representation of the amplified region and primers locations in the ND5 region of mtDNA. nt: nucleotides. The sequences of the primers are in the text.

A total of 878 bp of the ND5 sequence from each sample was used for phylogenetic analyses. This region is a useful mtDNA marker, used in previous studies to analyze inter- or intraspecific relationships of butterflies (ex. Makita *et al*. 2000; Kato & Yagi 2004). All sequences were aligned automatically using the Se-Al Sequence Alignment Program v1.d1. software (Rambaut 1996). Identical sequences were treated as a single operational unit (OTU). To test for saturation in base substitutions, we plotted uncorrected p-distance against the number of transitions and transversions (Fig. 3), based on calculations using PAUP* 4.0b (Swofford 2002).

Phylogenetic trees were constructed by Maximum Parsimony (MP) and Neighbor joining (NJ) methods using the program PAUP* 4.0b, and Bayesian analysis using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). The MP analyses were performed using a heuristic search with the TBR branch-swapping algorithm and 1000 random-addition replicates. The Kimura two-parameter model was used for analyses with the NJ method. The robustness of the branches was tested by bootstrap analyses (Felsenstein 1985) with 1000 and 100,000 replications for the MP and NJ methods, respectively. For Bayesian analysis, we applied the best-fit model (GTR+I+G) chosen by a hierarchical likelihood ratio test using MrModeltest 2.1 (Nylander 2004). Two runs with four chains of Markov chain Monte Carlo (MCMC) iterations were performed for 1,000,000 generations, keeping one tree every 100 generations. The first 25 % of the generations were discarded as burn-in and the remaining trees were used to calculate a 50 % majority-rule tree and to determine the posterior probabilities of the individual branches. The standard deviation for the two MCMC iteration runs was below 0.01, indicating convergence. The conclusive phylogeny was deduced from the Bayesian tree with the posterior probabilities, with the addition of the bootstrap values produced by the NJ and MP analyses.

We constructed a linearized NJ tree based on Kimura’s two-parameter model and estimated divergence times using the MEGA4 software (Tamura *et al*. 2007). The molecular clock hypothesis was tested by using Tajima’s relative rate test (Tajima 1993) to confirm the consistency of substitution rate among lineages. Divergence time estimations using the mitochondrial ND5 region have been reported by Kato and Yagi (2004). On the basis of the Kimura's two-parameter distance value (0.01D = 1.8 Myr), we deduced the nucleotide substitution rate of 5.56 x 10^{-9} per site per year.

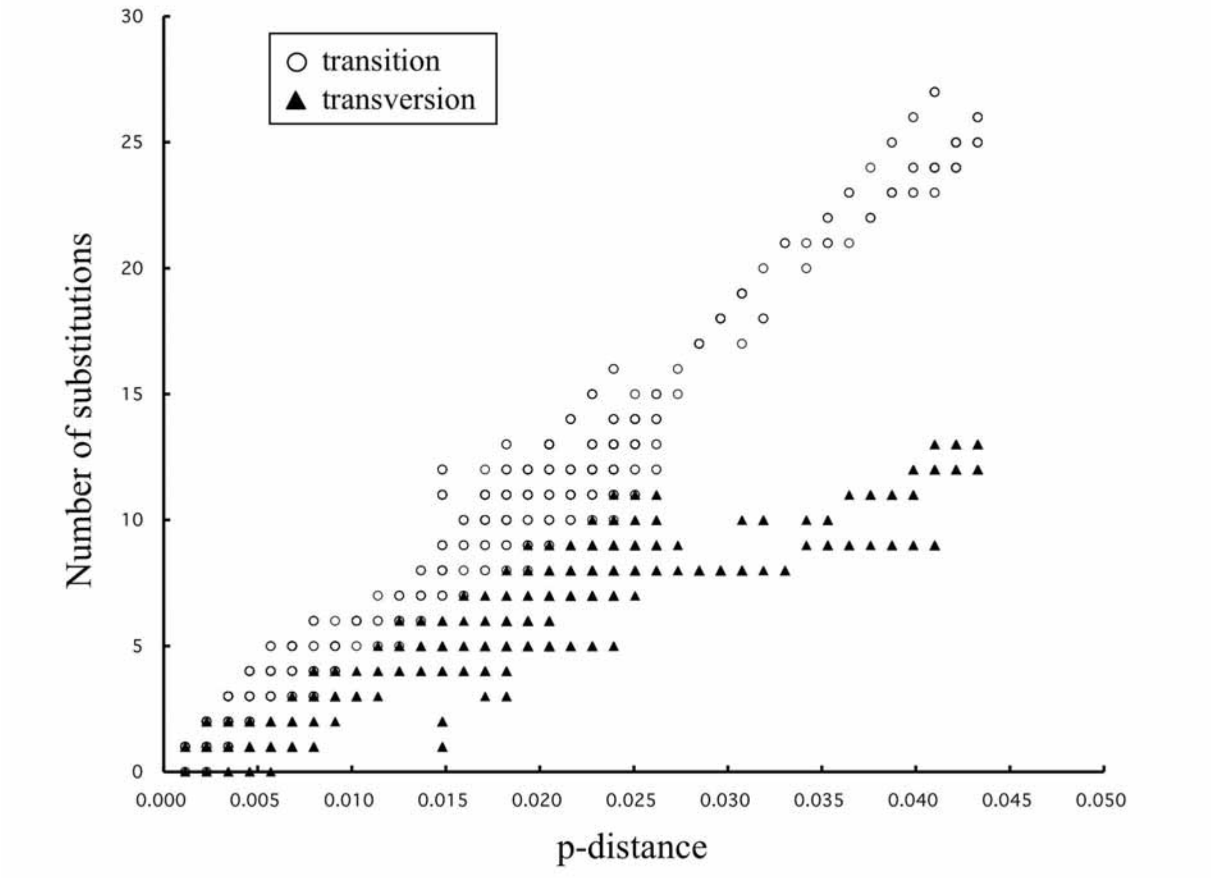


FIGURE 3. Plots of uncorrected p-distance against the number of transitions and transversions.

Results and Discussion

In this study, neither deletions nor insertions were found in the determined ND5 nucleotide sequences. Using a total of 44 OTUs, excluding identical sequences, we plotted the rate of uncorrected p-distance against the number of transitions and transversions. As a result, the scatter plots were represented as almost linear, so that saturation in base substitutions was not observed (Fig. 3). The G+C contents of the sequences ranged between 16.2 % and 18.2 % (average of the 44 OTUs: 17.2 %), and the range excluding the four out-group OTUs is from 16.6 % to 18.2 % (average of the 40 OTUs: 17.2 %) (Table 1). The values seem to be nearly constant in *Zizina*. Most nucleotide substitutions were found at the codon synonymous positions. The results suggest that the base composition bias and multiple nucleotide substitutions have no apparent effects on the phylogenetic trees. For this reason, the sequences were considered as suitable for the present analyses.

The phylogenetic trees showed almost the same topologies. However, in the trees produced by the MP and Bayesian methods, the clade composed of *Z. oxleyi* branched out earliest among species of the *Zizina*, followed by the clade comprising the two subspecies *emelina* and *thibetensis* of *Z. otis*. On the other hand, in one tree generated by the NJ method, the former clade was regarded as the sister group to the latter. The phylogenetic tree resulting from the Bayesian analysis is presented in Figure 4. In the following subsections, we reexamine the current classification and discuss the monophyly of each taxon and the interspecific relationships in *Zizina* based on this topology.

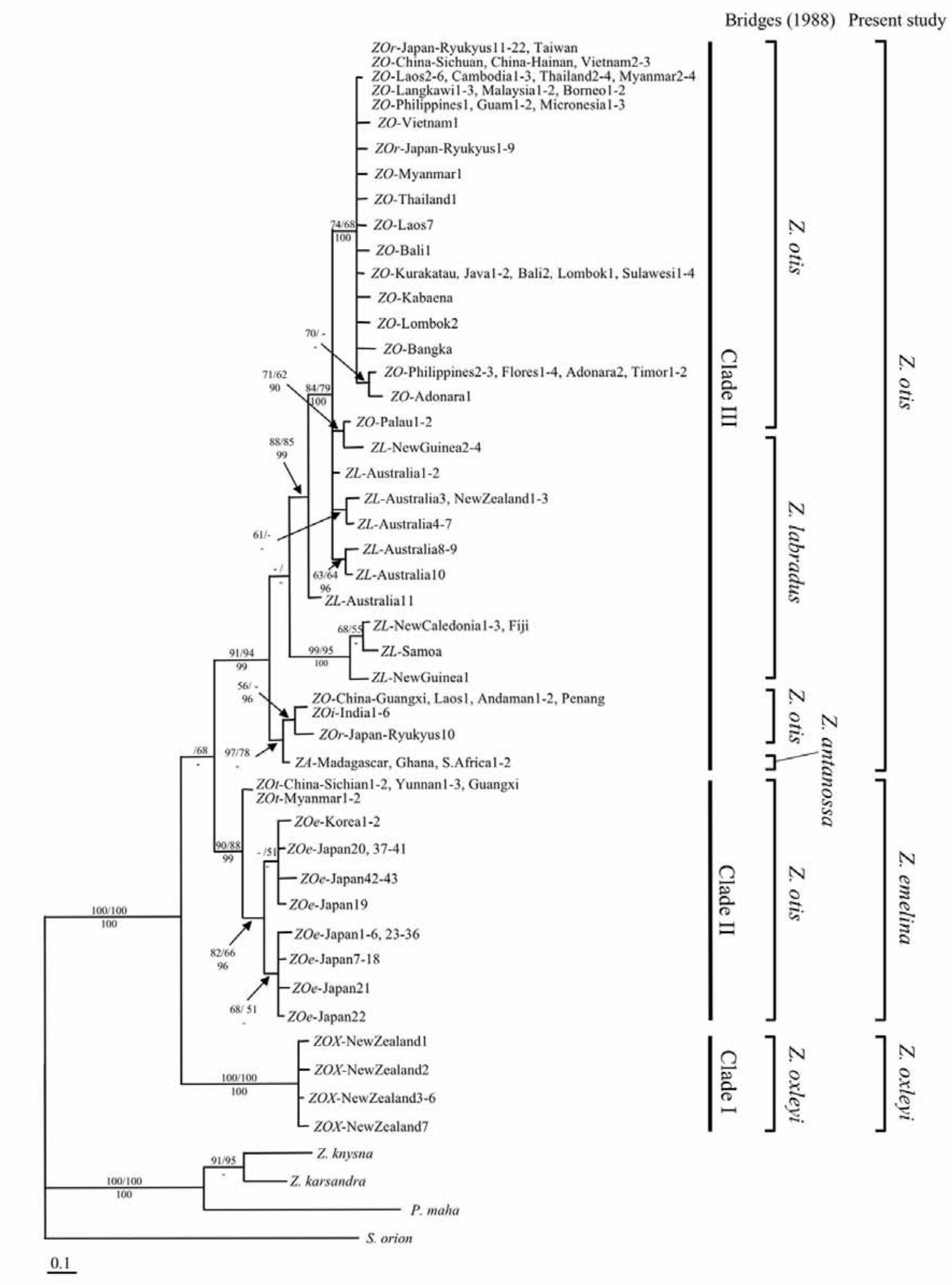
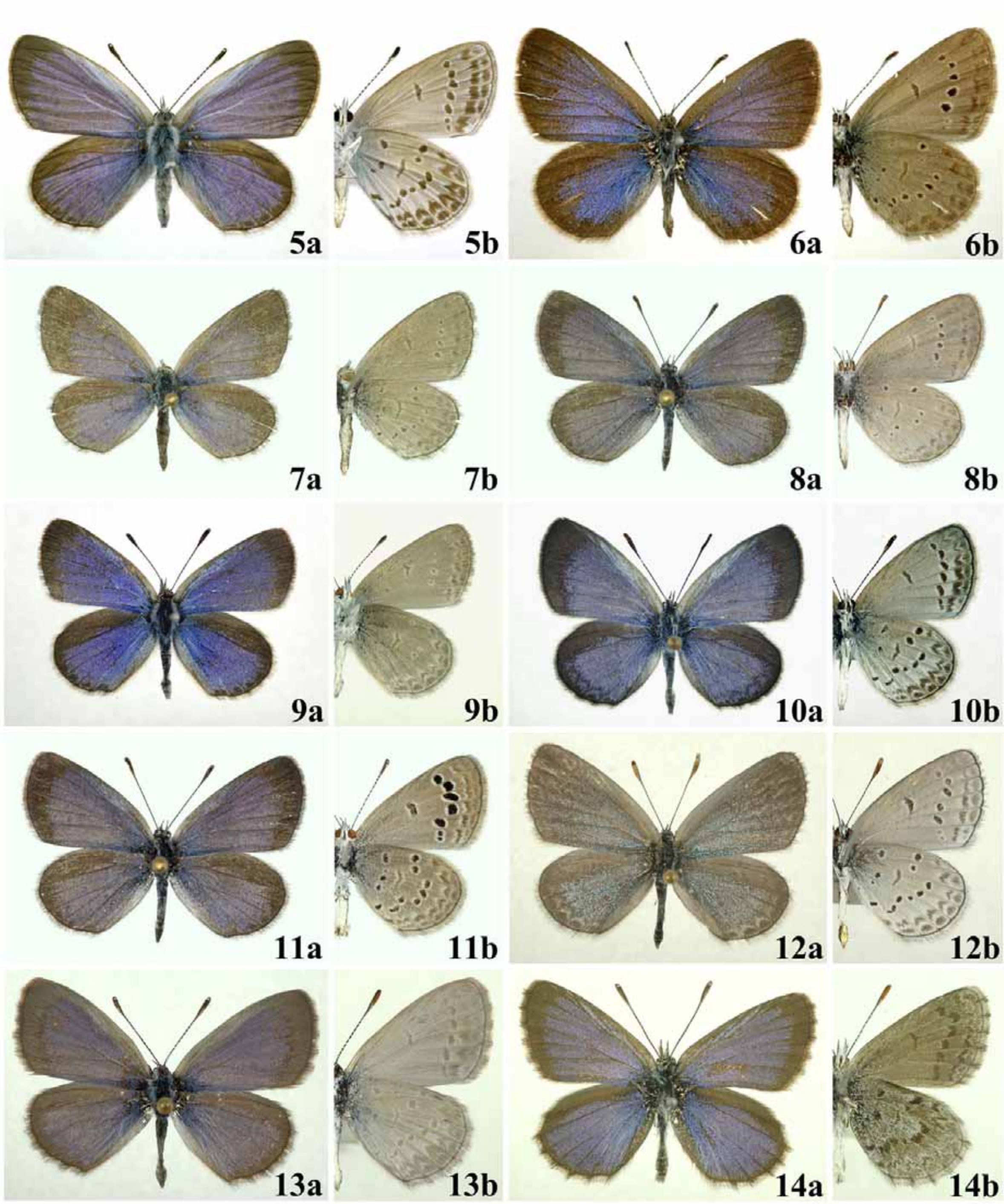


FIGURE 4. Phylogeny of species of *Zizina*. The topology shows the Bayesian tree based on 878 bp of mtDNA using the program MrBayes 3.1.2. Identical sequences were treated as a single OTU. Numbers indicate bootstrap values from NJ (top left) and MP (top right) analyses, and posterior probabilities from Bayesian analysis (bottom). Only bootstrap values >50 % and Bayesian posterior probabilities >90 are shown. Branch lengths represent nucleotide substitutions per site. The classification of Bridges (1988) and the present study are shown on the right.



FIGURES 5-14. Male wings of species and subspecies of *Zizina* (a: upperside; b: underside). The classification follows the present study (See also Results and Discussion in text). 5, *Z. emelina emelina*, Ono City, Hyogo Pref., Japan; 6, *Z. emelina thibetensis*, Zhanhe, Yunnan, China; 7, *Z. otis otis*, Jingxi County, Guangxi, China; 8, *Z. otis otis*, Havelock Is., South Andaman Iss., India; 9, *Z. otis riukuensis*, Daito Is., Okinawa Pref., Japan; 10, *Z. otis* ssp., Sikka, Flores, Indonesia; 11, *Z. otis indica*, Mysore, Karnataka state, India; 12, *Z. otis antanossa*, Aburi, Ghana; 13, *Z. otis labradus*, Cairns, Australia; 14, *Z. oxleyi*, North Canterbury, New Zealand. Magnification: 1.8 x.

1. Monophyly and taxonomic status of **Zizina** *species*

As shown in Figure 4, the monophyly of *Zizina* was strongly supported with high values of 100 % each (NJ, MP and Bayesian). Subsequently, this genus diverged into the following three independent clades: 1) *Z. oxleyi* (clade I), 2) *Z. otis emelina* + *Z. emelina thibetensis* (clade II), 3) *Z. labradus* + *Z. antanossa* + the remaining *Z. otis* (clade III). Of them, *Z. otis* and *Z. labradus* were regarded as polyphyletic. Although *Z. oxleyi* and *Z. antanossa* were represented as monophyletic, the phylogeny indicates that the specific status of the latter is suspicious. Thus, taxonomic re-examination at the specific level was necessary.

Clade I. The monophyly of clade I, composed of only *Z. oxleyi*, is strongly supported with very high values of 100 % each (NJ, MP and Bayesian; fig. 4). The genetic distance between this clade and clade II or III is very large (2.7-4.1 %) in the ND5 region of mtDNA, and four types with single nucleotide polymorphism were detected in clade I.

Zizina oxleyi is easily distinguishable from the other congeneric species in having unique wing markings (Figs 5–14), which are quite similar to those of a copper butterfly, *Antipodolycaena boldenarum* (White), occurring in New Zealand. Moreover, it differs from a sympatric *Zizina* species, *Z. labradus*, in having the male genital valva with a stouter process on the distal margin and two long subbasal bristles on the ventral margin (Figs 22–23). There are also distinctive morphological difference in the eggs and larvae of *Z. oxleyi* and *Z. labradus* (Gibbs 1980).

In previous studies, *Z. oxleyi* has often been treated as a subspecies of *Z. labradus* (or *Z. otis*), because there is the possibility that the two forms would be capable of interbreeding and morphological intermediates of the two have occasionally been observed in overlapping zones. However, further study was deemed necessary to establish whether this is a reasonable interpretation (Gibbs 1980). We analyzed seven specimens of *Z. oxleyi* captured in the sympatric area of the two forms. Two of them showed intermediate characters *sensu* Gibbs. Our genetic analyses show that *Z. oxleyi* is not related to *Z. labradus*, nor has introgression of mtDNA by hybridization occurred. It could be considered that copulation between *Z. oxleyi* and *Z. labradus* is difficult, or that successive interbreeding for several generations is not likely.

Clade II. Clade II, comprising *Z. otis emelina* (de L'Orza, 1869) from Japan and South Korea and *Z. otis thibetensis* (Poujade, 1885) from SW China and northern Myanmar, was strongly supported with high values of 90 % (NJ), 88 % (MP) and 99 % (Bayesian) (Fig. 4). Although *Z. o. emelina* and *Z. o. thibetensis* each also formed robust subclades with high values, the genetic distance between the two was comparatively small (0.3–0.6 %).

In the male wings, *Z. o. thibetensis* differs from *Z. o. emelina* in having a very broad marginal border on the upperside and a dark ground color on the underside (Figs 5–6). However, the two subspecies share the following characters: 1) large in size, 2) rows of post-discal, submarginal and marginal black dots on the wing underside expanded. Also, the male genital differences between the two are quite small (Figs 15–16). Thus, the monophyly of clade II is in no doubt.

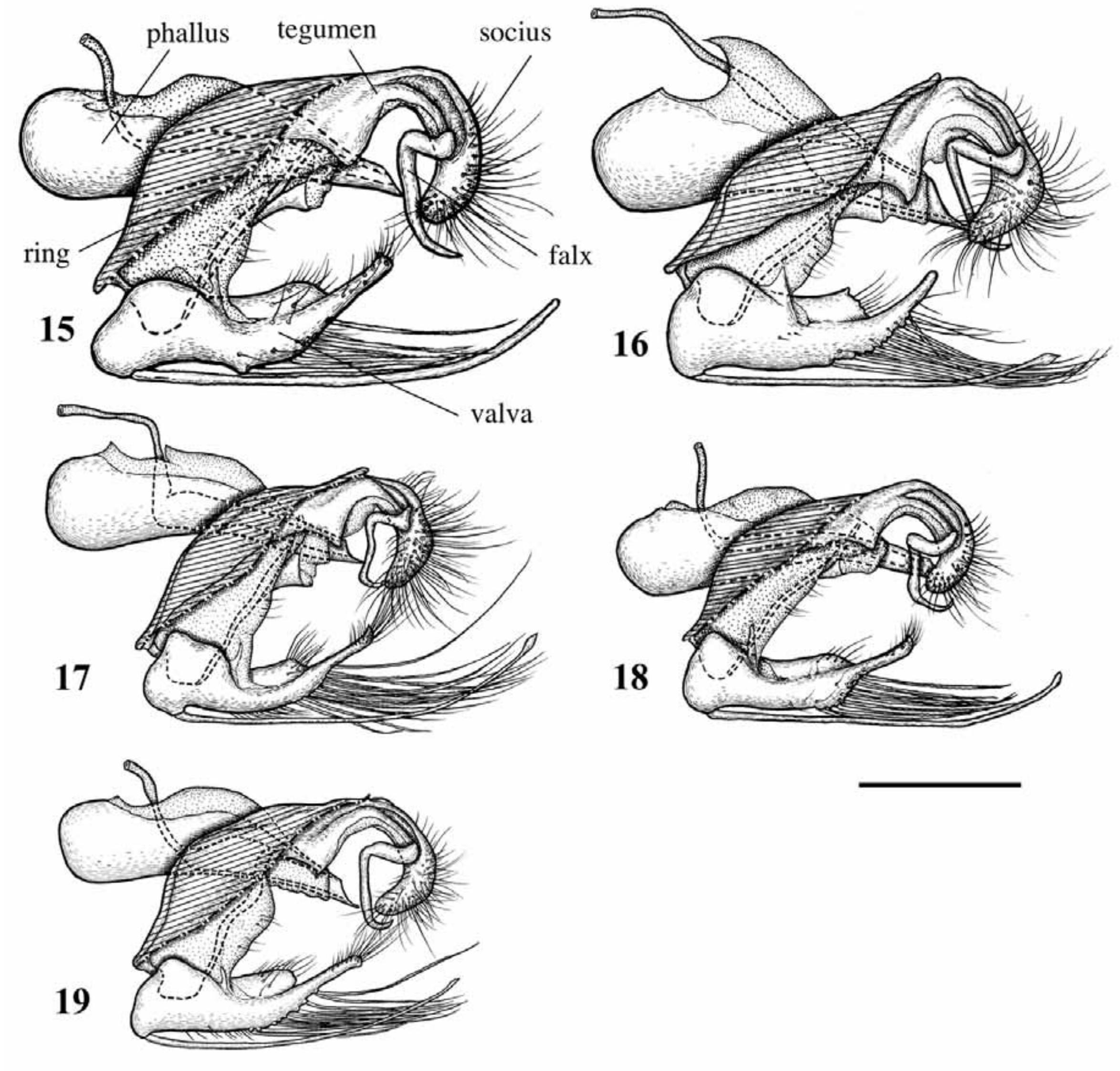
In the present analyses, the populations comprising *Z. o. emelina* and *Z. o. thibetensis* were found to be phylogenetically distant from the remaining *Z. otis* populations including the nominotypical subspecies (Fig. 4). The former populations differ from the latter populations, not only in size and wing markings (Figs 5–11), but also in the male genitalia, which are larger (1.3-1.5 times), the falx is rather weakly curved, and the valva is comparatively broader and more weakly swollen basally (Nishimura 2006; Yago 2007; figs 15–20). The differences in male genitalia are detected in many functionally important structures. In particular, the difference in the male genital falx suggests that the two populations would be incapable of copulation.

For these reasons, the populations comprising *Z. o. emelina* and *Z. o. thibetensis* are considered as a species distinct from *Z. otis*. Because the date of publication of *emelina* precedes that of *thibetensis*, this species should be treated as *Z. emelina* according to the Principle of Priority in the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature 1999).

Clade III. Clade III was firmly supported with values of 91 % (NJ), 94 % (MP) and 99 % (Bayesian) (Fig. 4). However, this clade is complex, nested by three currently recognized species, namely *Z. antanossa*, *Z. labradus* and *Z. otis* but excluding *Z. o. emelina* and *Z. o. thibetensis* that form clade II. Of these species, *Z. labradus* and *Z. otis* were regarded as polyphyletic.

The monophyly of *Z. antanossa* was strongly supported, because no genetic difference among our specimens from Madagascar, Ghana and South Africa was observed, even though they are geographically apart. The cluster, including *Z. antanossa*, mainly consists of the Indian *Z. otis indica*. Not only was this cluster supported with relatively high values of 97 % (NJ) and 78 % (MP), but also the genetic distances among *Z. antanossa* and the others of the cluster were quite small (0.2–0.3 %) (Fig. 4).

The male genitalia of *Z. antanossa* differ distinctly from those of the Oriental *Z. otis* occurring further east than eastern India (Figs 17-19, 21). However, the former are almost identical to those of *Z. o. indica* from Peninsular India, and share the following characters: 1) valva shorter, more robust and sickle-shaped, 2) valvular hairs on distal portion sparser, 3) phallus less swollen on central portion, suprazonal portion shorter (Figs 20-21). Based on the male genital morphology, some have treated *Z. o. indica* as a distinct species, *Z. indica* or *Z. antanossa* (Stempffer 1967; D'Abrera 1978, 1980, 1986; Corbet & Pendlebury 1992), though *Z. o. indica* differs from *Z. antanossa* in having the male wing upperside as in the Oriental *Z. otis* and much larger postdiscal black spots on the forewing underside (Figs 7-12). Our phylogenetic results were almost concordant with the male genital characters, but the cluster composed mainly of *Z. o. indica* also included samples of the Oriental



FIGURES 15-19. Male genitalia of *Zizina* species and subspecies (lateral views). The classification follows the present study. 15, *Z. emelina emelina*, Ono City, Hyogo Pref., Japan; 16, *Z. emelina thibetensis*, Leshan City, Sichuan, China; 17, *Z. otis otis*, Jingxi County, Guangxi, China; 18, *Z. otis riukuensis*, Okinawa Is., Okinawa Pref., Japan; 19, *Z. otis* ssp., Sikka, Flores, Indonesia. Scale bar: 0.5 mm.

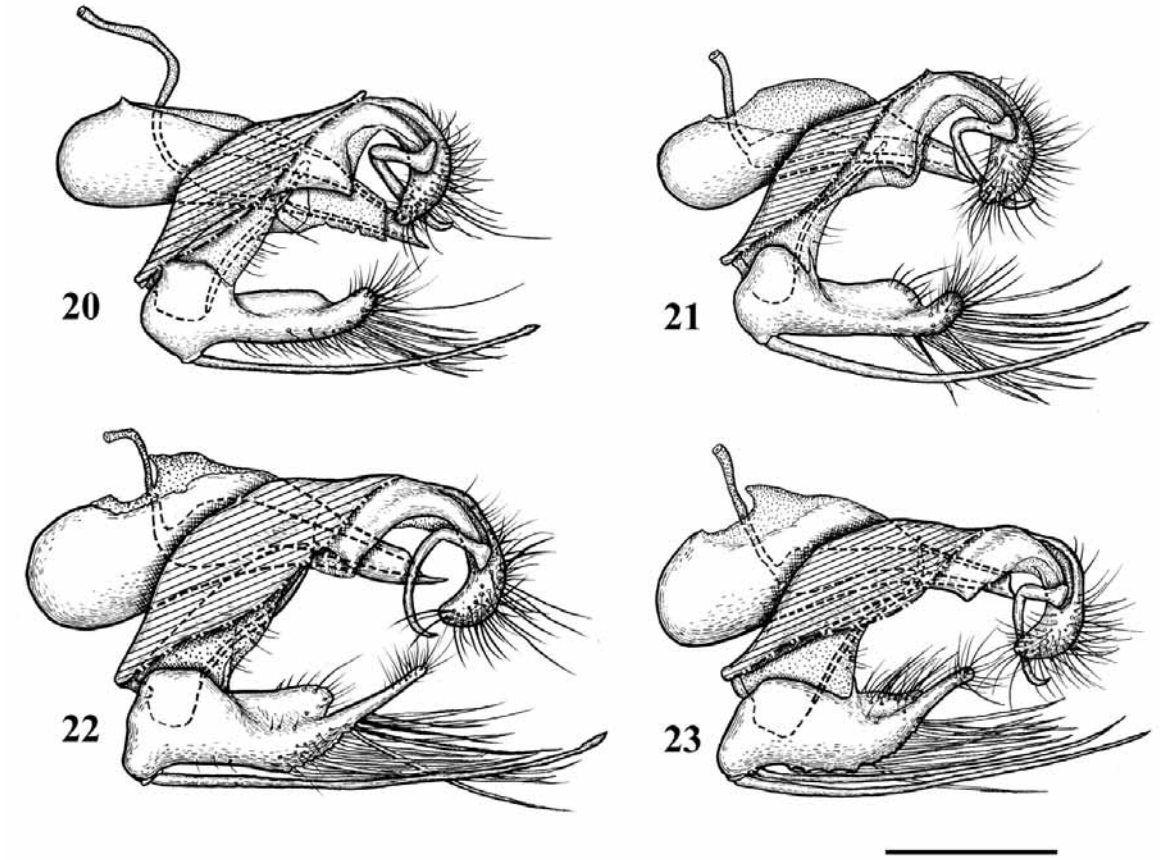
Z. otis from Andaman, Penang Is. (Malaysia), Laos, South China and Ryukyu Islands (Japan), and moreover, the other samples from Laos, South China and Ryukyu Islands served as factors of another cluster formed from the remaining *Z. otis* (Fig. 4).

When hybridization between species occurs, it is known that the true phylogenetic tree may not be induced due to introgression of mtDNA through maternal inheritance (Sota & Vogler 2001; Sota *et al.* 2001). Additionally, the sterility or low viability of the hybrid is often limited to the heterogametic sex (Haldane's rule) (Coyne 1985; Lukhtanov *et al.* 2005). As for lepidopterans, such as butterflies, even if interbreeding happens, since the females are heterologous in their sex chromosomes, their fertility and viability are low, therefore, gene flow of mtDNA via maternal inheritance between species is considered unlikely to occur. Moreover, lycaenids typically exhibit mate choice for appropriate conspecific wing coloration (Bernard & Remington 1991). Females of polyommatine species mate only once, so that heterospecific matings that do not produce viable offspring are strongly selected against (Drummond 1985; Lukhtanov *et al.* 2005), which should also hold true for *Zizina*. Thus, differences between the eastern and western populations of *Z. otis*, of which the boundary is in eastern India, may have been acquired in the process of speciation (incipient species). Judging from the high frequency of gene flow, however, our phylogeny indicates that they have not yet reached reproductive isolation and that gene exchange still takes place. Thus, *Z. antanossa* (and *Z. o. indica*) is here regarded as a subspecies of *Z. otis*, though this taxonomic statement may be practically clarified only through breeding experiments.

Our phylogeny showed that *Z. labradus* was a polyphyletic group forming six independent clusters within clade III (Fig. 4). Of them, the first cluster supported with high values of 99 % (NJ), 95 % (MP) and 100 % (Bayesian) was composed of *Z. labradus* occurring in New Guinea, Melanesia and Polynesia. This cluster appears to have diverged quite early from the common ancestor of clade III. However, the other specimens from New Guinea are included in another cluster (mentioned below). The second cluster consists only of an Australian specimen, and forms a sister-group to the clade comprising the remaining *Z. labradus* and the Oriental *Z. otis*. This sister-group relationship is strongly supported with rather high values of 88 % (NJ) and 85 % (MP) and 99 % (Bayesian). The third to sixth clusters consist of *Z. labradus* from Australia, New Zealand and New Guinea together with *Z. otis* from Palau, and *Z. labradus* was represented as multiple branching. In addition, the clade consisting of these clusters and the Oriental *Z. otis* was supported with relatively high values of 84 % (NJ), 79 % (MP) and 100 % (Bayesian).

Although we re-examined the wing markings and male genitalia of *Z. labradus* in each cluster, distinct and stable morphological differences among the clusters were minimal. Therefore, we consider these as populations capable of interbreeding. Comparing *Z. labradus* with the Oriental *Z. otis*, the male genital differences between the two (Figs 17-19, 22) show a cline from the Oriental to Australian regions as follows: 1) falx gradually and gently curved, 2) valva tending to be broader throughout length, 3) distal process of valva becoming more slender. Thus, this molecular and morphological evidence indicates that *Z. labradus* and the adjacent *Z. otis* could probably interbreed with each other. In addition, the underside wing markings of *Z. labradus*, except for the obscure large black spots, are similar to those of *Z. emelina* and not the Oriental *Z. otis*. Judging from our phylogeny, these character states seem to be more plesiomorphic than apomorphic within *Zizina*.

The remaining *Z. otis*, excluding those that form other clades with *Z. antanossa* and *Z. labradus*, consists of populations that are widely distributed in SE Asia, from the Ryukyu Islands to Indochina and the SE Asian Islands, further past the Wallace Line and to the Lesser Sunda Islands. This cluster was supported with relatively high values of 74 % (NJ), 68 % (MP) and 100 % (Bayesian) (Fig. 4). The individuals included in this cluster are characteristic of low genetic distances (0-0.5 %), and differ from other *Zizina* species in the following characters: 1) small in size, 2) black dots on the wing underside much smaller and more reduced, 3) male genitalia distinctly smaller (about two thirds), 4) falx strongly hooked, 5) valva more swollen basally, 5) distal process of valva more slender (Nishimura 2006; Yago 2007; figs 5-23). As mentioned above, however, some other individuals, which are morphologically indistinguishable from the individuals of this cluster, are included also in the other clusters containing *Z. antanossa* and *Z. labradus*. Presumably, the Oriental *Z. otis* might be able to interbreed with any of the parapatric populations in clade III.



FIGURES 20-23. Male genitalia of species and subspecies of *Zizina* (lateral views). The classification followed the present study. 20, *Z. otis indica,* Mysore, Karnataka State, India; 21, *Z. otis antanossa,* Aburi, Ghana; 22, *Z. otis labradus,* Cairns, Queensland, Australia; 23, *Z. oxleyi,* Hanmer Springs, North Canterbury, New Zealand. Scale bar: 0.5 mm.

It is concluded that the genus *Zizina* is classified into three species, *Z. otis*, *Z. emelina* and *Z. oxleyi*. *Zizina labradus* and *Z. antanossa,* which have generally been treated as specifically distinct, are considered to be subspecies of *Z. otis*. The new classification and distributions of the *Zizina* species are as follows:

Genus *Zizina*

Z. otis [= *Z. antanossa, Z. labradus*]: China (type locality), Japan [Ryukyus], Taiwan, Indochina, India, Pakistan, Sri Lanka, Andaman, Philippines, Palawan, Mariana Islands, Malay Peninsula, Borneo, Sumatra, Indonesian Archipelago, New Guinea, Micronesia, Melanesia, Polynesia, Australia, New Zealand, Africa, Madagascar, Mauritius.
Z. emelina: Japan [except for Ryukyus] (type locality), South Korea, SW China, northern Myanmar.
Z. oxleyi: New Zealand (type locality).

2. Phylogeography and speciation of the genus Zizina

Divergence time estimations were made by constructing an NJ tree. Analysis based on Tajima's relative-rate test confirmed that a molecular clock could be hypothesized for our data set ($p < 0.05$). The molecular clock of 5.56×10^{-9} substitutions per site per year was applied (see Materials and Methods; fig. 24). *Zizina* is initially divided into the three large clades (I-III), and this divergence occurred about 2.5 Mya (million years ago). In other current molecular studies of lycaenids, the divergence time of recent speciation, based mainly on the COI region of mtDNA, ranges roughly from 0.1 to 3 Mya (Als *et al*. 2004; Kandul *et al*. 2004; Megens *et al*. 2004). Our estimated age of speciation in *Zizina* is within this range. This estimation clearly denies its previously hypothesized Gondwanan origin (120-160 Mya, Hall 1998; Metcalfe 1998) of this genus (Stempffer 1967; Kawazoé & Wakabayashi 1976). The present ice age is assumed to have started 40 Mya, and beginning around 3 Mya, the violent fluctuations between glacial and interglacial periods gradually occurred throughout the world (Lisiecki & Raymo 2005; Raymo *et al*. 2006). The start of the extreme climatic variation almost coincides with early divergences within the genus. Therefore, speciation in this genus could have rather been caused by adaptation to climatic variations. We hypothesize that the populations of the common ancestor to *Zizina* that adapted to temperate zones in the Northern Hemisphere gave rise to *Z. emelina* and in the Southern Hemisphere to *Z. oxleyi*. Those populations in the subtropical and tropical zones were ancestral to *Z. otis* (Fig. 25).

With respect to clade I, it must be mentioned that the divergence of the ancestral *Z. oxleyi*, that separated from the common ancestor to *Zizina*, occurred quite early and adapted to the temperate New Zealand (Figs 24-25). Four endemic lycaenine species (coppers) might also be examples of such speciation (Gibbs 1980; Yago & Saigusa 2000). It has been hypothesized that *Z. oxleyi* evolved through Gondwanan vicariance or by wind dispersal from Australia over the Tasman Sea (Gibbs 1980). Since our phylogenetic analyses preclude the former hypothesis, it would be reasonable to postulate that the ancestral *Z. oxleyi* dispersed across the water gap by intense wind or across land connected by lower sea levels during glacial periods of the ice age, and was isolated in New Zealand. As examples of enigmatic occurrences in polyommatine butterflies, there are *Udara blackburni* (Tuely) on the Hawaiian Islands and *Celastrina ogasawaraensis* (H. Pryer) on the Ogasawara Islands. The two species are endemic to ocean islands which had always been far from landmasses, but they are probably closely related to the SE Asian species of *Udara* or *Celastrina* (Shirôzu 1960; Eliot & Kawazoé 1983).

Zizina emelina, composing clade II, is represented by two geographically separated populations in the temperate zone; *Z. e. emelina* from the Japanese mainland and South Korea, and *Z. e. thibetensis* from the highlands of SW China to Myanmar (Figs 24-25). Other examples of lycaenid butterflies that show similar distribution patterns are *Fixsenia iyonis* (Ota & Kusunoki) and some thecline species (Inomata 1989; Odagiri 2007). While *Z. e. emelina* and *Z. otis* can be found parapatrically in the northern part of the Ryukyu Islands, *Z. e. thibetensis* and *Z. otis* appear to be separated only by altitude in SW China to Myanmar and are almost sympatric or parapatrically distributed in locations quite close to each other (Fig. 25; table 1).

In clade III, *Z. otis*, occurring mainly in tropical and subtropical zones, diverged into three subclades (III-1, 2, 3) (Fig. 24). Firstly, subclade III-1 consists of *Z. o. labradus* mainly from Melanasia to Polynesia (Fig. 24). It is possible that the common ancestor of *Z. otis* was isolated for a long time on some ocean islands, diverged genetically, and dispersed, likely with tropical storms, to its surrounding islands, with some movement into New Guinea. Gibbs (1980) also mentioned that the presence of *Z. o. labradus* in Melanesia to Polynesia indicated that they have the vagility that allows them to occasionally cross the seas.

Subclade III-2 consists mainly of two subspecies, the African *Z. o. antanossa* and the Indian *Z. o. indica* (Fig. 24). We infer that the common ancestor of the two taxa was isolated from that of the remaining *Z. otis* lineages for a long period, perhaps due to climatic variation and/or a geographical barrier, leading to considerable genetic divergence and male genital differences (Figs 17-22, 24). The common ancestor was recently divided into two populations by the vast dry area extending from northeast Africa to the Near and Middle East. One evolved into the extant *Z. o. antanossa* and rapidly spread throughout Africa, whereas the other became the Indian *Z. o. indica*, merged with the remaining *Z. otis* from Indochina to Malay, and interbred again at the boundary (Figs 24-25). Despite their genetic similarity, the male upperside coloration of *Z. o. antanossa* differs markedly from the other *Z. otis* populations including *Z. o. indica* (Figs 8-13). This may be the result of a bottleneck effect, i.e. the ancestral *Z. o. antanossa* was isolated to a small area, where genetic mutations resulting in their unique wing phenotype accumulated.

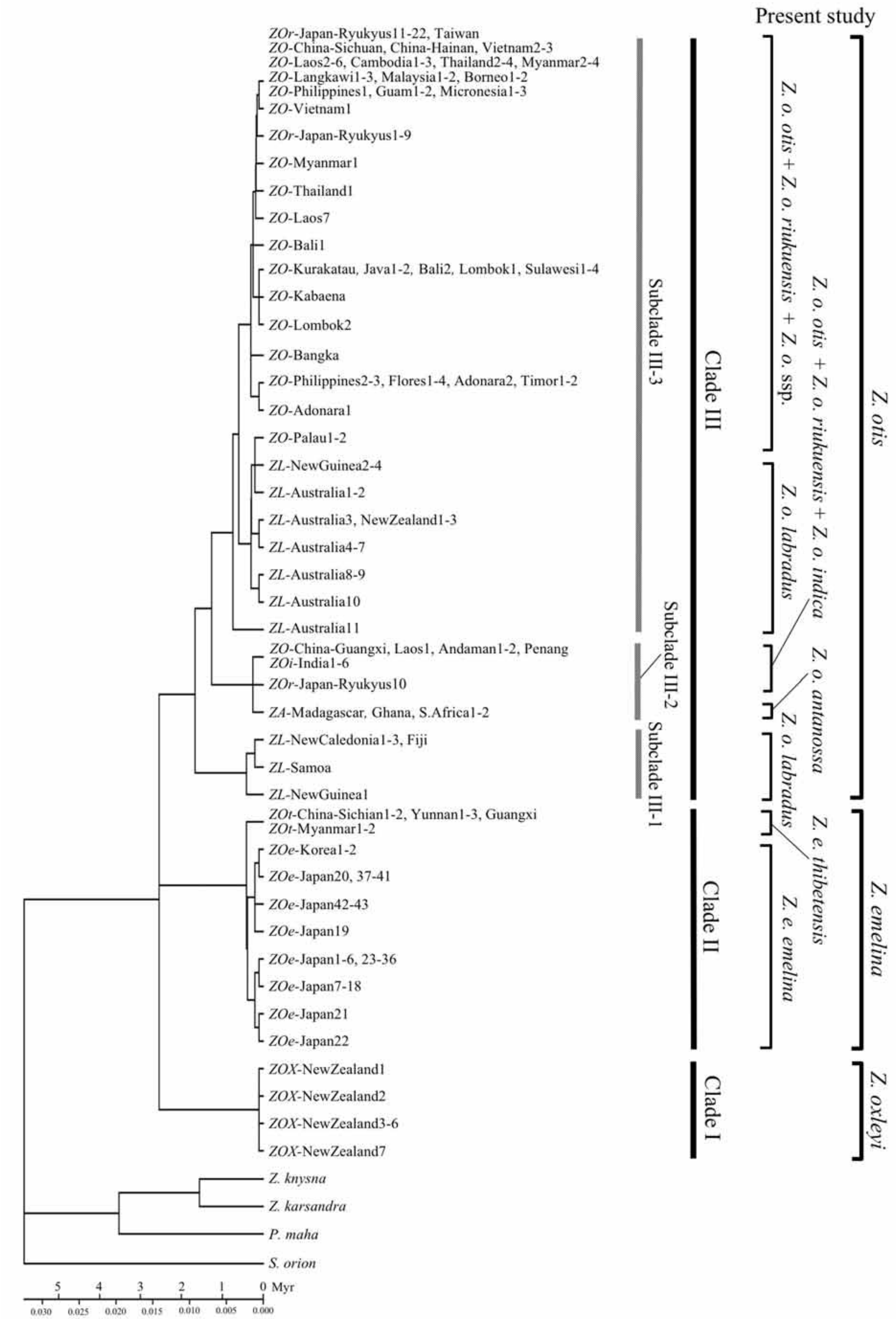


FIGURE 24. Linearized NJ tree based on Kimura's two-parameter model using ND5 nucleotide sequences from mtDNA. Identical sequences were treated as a single OTU. The molecular clock of 5.56 x 10^{-9} substitutions per site per year (0.01D=1.8 Myr) was applied for the divergence time estimations. The right hand side indicates our classification.

Finally, subclade III-3 is constituted mainly by *Z. otis* populations from the Oriental to Australian regions, excluding Melanesia and Polynesia (Fig. 24). The Australian samples examined in our study are few; nevertheless, since 1) the Australian populations are continuously branched in the early phase, 2) genetic divergences among the Oriental populations are quite small, 3) a cline from the Australian to Oriental populations is observed in male genitalia, we propose that the common ancestor to this subclade was, for a long period, isolated in the Australian region. Subsequently there was dispersion to the Oriental tropical zone, with further spread occurring over a relatively short period of time. Thus, the evolution of this subclade may be phylogeographically interpreted as having been formed by the progression rule (Hennig 1966) in the direction from south to north (Figs 24-25).

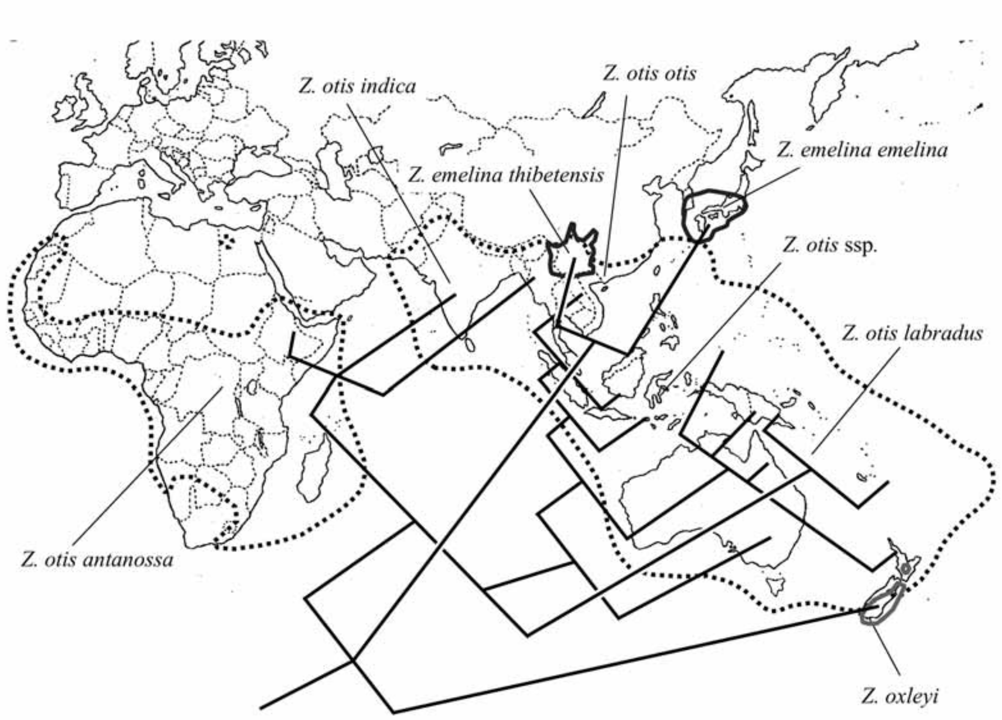


FIGURE 25. Phylogeography of species of *Zizina* inferred from the present study.

Changes in the feeding habits of the larval stages, in response to changing climates, may also have influenced speciation events and the geographical distribution of species in *Zizina*. *Alysicarpus vaginalis*, *Desmodium triflorum*, *Indigofera spicata*, *Kummerovia striata*, *Tribulus terrestris*, and several introduced legumes have been recorded as the main hostplants of *Z. otis* in the Oriental region (Robinson *et al*. 2001). *Lotus japonicus* and *Parochetus communis* (unpublished data) are recorded as the hostplants of *Z. e. emelina* and *Z. e. thibetensis*, respectively, which occur parapatrically with *Z. otis*, although *Z. e. emelina* also occasionally feeds on *K. striata* (Fukuda *et al.* 1986). The larvae of *Z. otis* were found to grow poorly when we fed them *L. japonicus* (data not shown). *Zizina o. labradus* from Australia and New Zealand are known to feed on almost any clover, trefoil or medic, such as *Trifolium*, *Medicago* and *Lotus* (Gibbs 1980; Common & Waterhouse 1981). Although the larvae of *Z. oxleyi* feed on some clovers and lotus as in *labradus*, it is likely that native brooms of *Carmichaelia* and the scarce native pea, *Swainsona novae-zelandiae*, were its original hostplants prior to the introduction of the above-mentioned pasture legumes (Gibbs 1980). The fact that the original hostplants differ between these two species suggests that the ancestors of each speciated through adaptation to both the climate and vegetation in their respective localities.

In New Zealand, *Z. o. labradus* is currently widespread in the North Island and the northern and western regions of South Island, whereas *Z. oxleyi* occurs in the eastern and southern areas of South Island and locally in the central region of North Island. According to Gibbs (1980), there were no records for *Z. o. labradus* before European immigrants began spreading their legume pastures in the late 19th century, and *Z. oxleyi* seems to have been the common species in both islands until *Z. o. labradus* expanded its distribution. The expansion of *Z. o. labradus* could have occurred in two possible ways; one in which it was introduced by humans in animal fodder from Australia, and the other by wind dispersal (Gibbs, 1980). If introduction by humans had taken place, it is expected that there would be no genetic distance between Australian and New Zealand *Z. o. labradus* populations. Supporting this scenario, the ND5 sequence of one of the eleven Australian individuals was completely concordant with those of the New Zealand exemplars (Fig. 24). If the distance between Australia and New Zealand was within their flying ability, a native *Z. oxleyi* would not have occurred in New Zealand today, though *Z. o. labradus* are known to disperse occasionally with the aid of seasonal winds in Australia (Smithers 1985; Faithfull 1987; Dingle *et al*. 1999). For these reasons, it is probable that the New Zealand *Z. o. labradus* is a recently anthropogenically introduced species from Australia or its surroundings, causing reduction of the geographical range of *Z. oxleyi*. It serves as an instructive case for the conservation of biodiversity in warning us about the dangers of introducing butterflies into habitats already occupied by congeners.

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