Phylogeography of the Delias hyparete species group (Lepidoptera: Pieridae): complex historical dispersals into and out of Wallacea

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The unique and highly endemic fauna of Wallacea has been extensively examined; however, the diversification of a single animal lineage in Wallacea has not yet been studied in detail. The Delias hyparete (Linnaeus) species group is distributed in the Oriental and Australian regions as well as throughout Wallacea (i.e. North Maluku, South Maluku, Sulawesi and Lesser Sunda), with the highest species diversity occurring in Wallacea. The present study reconstructed the phylogeny and estimated the between- and within-species divergence of the D. hyparete group using two genes: mitochondrial NADH-dependent dehydrogenase subunit 5 (ND5) and nuclear elongation factor 1 alpha (EF1-α). Two out of five clades were associated with Lesser Sunda, and the remaining three clades were associated with North Maluku, South Maluku and Sulawesi, respectively. Ancestral area analyses and molecular dating suggested five colonization events into Wallacea at various times with the Australian and Oriental regions inferred as the geographical origins; Lesser Sunda may have been colonized twice. Two range expansion events from Lesser Sunda to Greater Sunda in the recent past and an older dispersal passing through Wallacea towards the Oriental region have also been inferred. The species diversity of this butterfly group in Wallacea appears to have developed due to non-sympatric speciation events. Complex historical dispersals into and out of Wallacea inferred in the D. hyparete group reinforce the view that animals move across Wallace’s and Lydekker’s Lines more frequently than classical assumptions, and these complex dispersals may have largely contributed to the high biodiversity and endemism evident in Wallacea.


INTRODUCTION

Wallacea comprises a group of islands surrounding the Banda Sea, Indonesia, and is a biogeographical region between the Oriental and Australian regions (Wallace, 1867a; reviewed by Michaux, 2010; Lohman et al., 2011; see also Escalante, 2016). While the definition of Wallacea has historically been complicated, we here defined it as a region delineated by Wallace’s Line along the Makassar and Lombok Straits and Lydekker’s Line surrounding the eastern edge of the Banda Sea (Fig. 1; Lohman et al., 2011). The fauna of Wallacea is a composite of Asian and Australian/New Guinean taxa (reviewed by Michaux, 2010). The biodiversity of Wallacea islands is often high, and its fauna has a high level of endemism (Michaux, 2010; Lohman et al., 2011; Ng et al., 2016). Wallacea is mostly composed of oceanic...
islands which have not been connected to continents (Lohman et al., 2011). Therefore, ancestors of animal lineages now endemic to Wallacea immigrated via overseas dispersal from the Oriental and/or Australian regions. Beck et al. (2006) biogeographically examined the diversity of hawkmoths in Wallacea, and suggested that dispersal was a relatively important determinant of fauna similarities among the islands there.

Until recently, it was often implicitly assumed that historical dispersal into Wallacea was rare (Bellemain & Ricklefs, 2008; Balke et al., 2009). However, several studies demonstrated more frequent and complex historical dispersals across Wallace’s and Lydekker’s Lines. Birds of the genus *Pericrocotus* dispersed out of the Australian region into Greater Sunda and other Asian regions through Wallacea (Jönsson et al., 2008). A group of diving beetles historically jumped across Wallace’s and Lydekker’s Lines during a single main event (Balke et al., 2009). Lohman et al. (2011) reviewed molecular biogeographical studies for Wallacea, and indicated that Wallace’s and Lydekker’s Lines are permeable boundaries for fauna.

Figure 1. Map of Wallacea showing some of the collection localities of the *D. hyparete* group used in the present study. Solid dots, open dots, rectangles, triangles, crosses and rhombi represent the Oriental region, Australian region, Sulawesi, the Lesser Sunda Islands, North Maluku and South Maluku, respectively. 1, Leyte [*D. hyparete*]; 2, Thailand [*D. hyparete*]; 3, Nias [*D. hyparete*]; 4, Belitung [*D. hyparete*]; 5, East Java [*D. periboea*]; 6, Kangean [*D. periboea*]; 7, Bali [*D. sambawana, D. hyparete* and *D. periboea*]; 8, Nusa Penida [*D. hyparete*]; 9, Lombok [*D. sambawana and D. periboea*]; 10, Sumbawa [*D. sambawana*]; 11, Sumba [*D. fasciata*]; 12, Flores [*D. sambawana*]; 13, Alor [*D. sambawana and D. periboea*]; 14, Wetar [*D. timorensis*]; 15, Damar [*D. timorensis*]; 16, Timor [*D. eileenae and D. timorensis*]; 17, Moa [*D. timorensis*]; 18, Babar [*D. timorensis*]; 19, Tanimbar [*D. timorensis*]; 20, Centre Sulawesi [*D. rosenbergii*]; 21, Muna [*D. rosenbergii*]; 22, Peleng [*D. mitisi*]; 23, Halmahera [*D. poecilea*]; 24, Bacan [*D. poecilea*]; 25, Obi [*D. edela*]; 26, Buru [*D. ceneus*]; 27, Ambon [*D. ceneus*]; 28, Biak [*D. euphemia*]; 29, Aru [*D. mysis*]; 30, New Guinea (Fakfak) [*D. lara*]; 31, New Guinea (Timika) [*D. lara*]. The following localities of samples are not shown. New Guinea [*D. doylei*], Australia [*D. argenthona* and *D. mysis*], India [*D. hyparete* and *D. eucharis*], Nepal [*D. hyparete*] and Andaman [*D. hyparete*].
A few groups of flightless weevils were suggested to have transgressed Wallace’s and/or Lydekker’s Line(s) repeatedly (Tänzler et al., 2014, 2015; Toussaint et al., 2015). The ancestral area reconstruction analysis on other insects suggested repeated colonization into Wallacea (carpenter ants Camponotus maculatus and its allies: Clouse et al., 2015; diving beetles of the genus Platynectes: Toussaint et al., 2016; burying beetles of the genus Nicrophorus: Toussaint & Condamine, 2016). Thus, Wallacean islands may have been colonized by animals more frequently than classically assumed. Wallacean islands may have served not only as the recipients of colonizers from continents but also as a source of emigrants to continents (reverse colonization, Bellemain & Ricklefs, 2008; Grismer et al., 2016). However, empirical studies critically examining these hypotheses remain limited both in number and in taxonomic range. The historical processes responsible for the formation of endemic species in Wallacea have not yet been sufficiently examined (Braby & Pierce, 2007). The diversification histories of more groups of animals diversifying within Wallacea warrant further study.

Butterflies have long been a model group to understand the pattern and history of faunal diversification in the Indo-Pacific Archipelago including Wallacea (reviewed by Braby & Pierce, 2007; Condamine et al., 2015; Toussaint & Balke, 2016). Recently, molecular phylogeographical studies have been shedding light on the detailed diversification history of Wallacean butterflies such as birdwing butterflies (Condamine et al., 2015), nymphalids (Müller et al., 2010; Toussaint & Balke, 2016) and pierids (Braby & Pierce, 2007; Müller et al., 2012). Müller et al. (2010, 2012) reported that Wallacea was not only a transitional zone between the Oriental and Australian regions, but also by itself important in the evolution of animals, such as butterflies. The genus Delias (Hübner) has the most species in the family Pieridae. Delias butterflies are distributed in South and Southeastern Asia, Wallacea, New Guinea, Australia and Melanesia, and are most diversified in New Guinea (Yagishita et al., 1993). The occurrence of many Delias species in the Oriental region, the Australian region and Wallacea makes the genus particularly attractive to investigate the historical biogeography in and around Wallacea (Braby & Pierce, 2007). The number of species in Delias has continued to increase since Wallace (1867b) first listed 57 species: c. 250 species in 22 species of species are currently recognized (Yagishita et al., 1993; Braby & Pierce, 2007; Müller et al., 2012). Morinaka et al. (2002) and Braby & Pierce (2007) examined phylogenetic relationships among the species groups of Delias. Braby & Pierce (2007) suggested that Delias originated on the Australian plate. This was later supported by the findings of a phylogenetic analysis of 138 Delias species (Müller et al., 2012). Braby & Pierce (2007) suggested at least seven dispersal events from the Australian region to the Oriental region across Wallacea. Müller et al. (2012) inferred that several species groups of Delias colonized Wallacea during the late Miocene and Pliocene, the early stages of the evolutionary history of the genus.

The present study focused on the Delias hyparete (Linnaeus) species group (Supporting Information, Fig. S1), which was suggested by Braby & Pierce (2007) to be a good model to look at historical dispersal across Wallacea. In the D. hyparete species group, 21 species have been identified to date, ranging from India in the west to the Solomon Islands in the east, and from China in the north to Australia in the south (Yagishita et al., 1993; Parsons, 1998; Braby, 2000; Davenport & van Mastigt, 2008). Delias hyparete and Delias eucharis (Drury) are distributed in mainland Asia, while the others are distributed in Australia, New Guinea, Melanesia and/or Wallacea. Ten out of the 21 species are endemic to or centred in Wallacea, covering the entirety of Wallacea (North Maluku, South Maluku, Sulawesi and Lesser Sundas; Fig. 1). Thus, the species diversity of the D. hyparete group is higher in Wallacea than in the Oriental or Australian region. In addition, Delias sambawana Rothschild and Delias periboea (Godart) attracted interest because they are distributed in Lesser and Greater Sundas, i.e. both sides of Wallace’s Line (Fig. 1). These latter two species provide us with the opportunity to phylogenetically examine the time and direction of their historical dispersal across Wallace’s Line. Therefore, this group is considered suitable for studying the diversification process of animals in Wallacea. Braby & Pierce (2007) assumed that the D. hyparete group dispersed only once across Wallacea to infer the minimum number of historical dispersals among the Oriental region, the Australian region and Wallacea; however, they also pointed that fine-scale phylogeny is required to infer the number and timing of historical dispersals within Wallacea and among Wallacea, the Oriental region and the Australian region for geographically widespread species-groups such as the D. hyparete group. Müller et al. (2012) used 14 species of this group in their molecular phylogenetic analysis of 138 Delias species, suggesting early dispersal events from Wallacea towards the Oriental region in the D. hyparete group. However, the diversification of the Wallacea-endemic species in the D. hyparete group has not been investigated in detail. Some Wallacean species were not sampled, and sequence variations within species were not examined by Müller et al. (2012).

The aim of the present study was to examine the history of colonization into/out of and diversification within Wallacea of the D. hyparete group using most known Wallacean species and many subspecies in the D. hyparete group. Molecular phylogenetic, molecular
dating and ancestral area analyses were combined to infer the route and timing of the historical dispersal of the Delias hyparete group into, out of and/or within Wallacea. We also updated the taxonomy of the Delias hyparete group based on the molecular phylogeny.

MATERIAL AND METHODS

The butterflies of most species of the genus Delias (Hübner) have brightly coloured markings on the underside of the wings, and wing markings often markedly vary among Delias species. The bright wing markings of Delias are considered to be aposematic to predators; Delias butterflies are considered unpalatable (Morishita & Yata, 1981; Braby & Nishida, 2010). The larval host plants of Delias species are, to the best of our knowledge, plants of Loranthaceae, their relatives in Santalales and Euphorbiaceae (Talbot, 1928–1937; Morishita & Yata, 1981; Stirpe, 1983; Corbet & Pendlebury, 1993; Parsons, 1998; Braby, 2006, 2012; Braby & Trueeman, 2006).

We sampled 18 of the 21 species in the Delias hyparete group (Table 1, Supporting Information, Fig. S1). The three species not used were Delias bagoe (Boisduval) from the Bismarck Islands, Delias schoenbergi Rothschild from the Solomon Islands and Delias aestiva Butler from the Northern Territory and north-western Queensland in Australia (Braby, 2014). In order to cover a large part of the species distribution range, we included as many subspecies as possible: five out of seven subspecies for D. periboea, six out of seven subspecies for Delias timorensis (Boisduval), all five subspecies for D. sambawana and seven out of 20 subspecies for D. hyparete (Table 1). When the previous (Müller et al., 2012) and present studies were combined, all known species of the Delias hyparete group, except for D. aestiva, were phylogenetically analysed: D. aestiva was recently recognized as a distinct species by Braby (2012, 2014), inhabits the Northern Territory and Queensland, Australia, and is close to Delias mysis (Fabricius). The outgroup species used were as follows: Delias eichhorni Rothschild & Jordan, Delias toxopei Roepke, Delias carstensziana Rothschild, Delias gabia (Boisduval), Cepora iudith (Fabricius), Aporia crataegi (Linnaeus) and Leucia cracta acuta Rothschild & Jordan. Sequence data for D. eichhorni, D. toxopei and D. carstensziana were previously published in Morinaka et al. (2002). Insects from countries other than Japan were obtained for years as dried specimens from Japanese amateur collectors/researchers of butterflies. Voucher specimens are kept at the laboratory of S. Morinaka (Table 1).

Based on their original descriptions, the names Delias poecilea (Vollenhoven) and Delias ceneus (Linnaeus) were used in the present study. However, these butterflies have been widely described as D. poecilea and D. caeneus, respectively, in most of the literature (Talbot, 1928–1937; D’Abrera, 1990; Yagishita et al., 1993).

The Delias species used in the present study were largely included in the previous study by Müller et al. (2012), but the two studies do not share sequence data. Total DNA was obtained from butterfly bodies or legs with the standard protocol using sequential extraction with phenol, phenol/chloroform (1:1, v/v), and chloroform followed by precipitation with ethanol. The precipitants were dissolved in 200 µL TE solution (i.e. 10 mM Tris–HCl and 1 mM EDTA). Using DNA solution as the template, the 924-base pair (bp) portion of the mitochondrial NADH-dependent dehydrogenase subunit 5 (ND5) gene and 1087-bp portion of the nuclear elongation factor 1 alpha (EF-1α) gene were amplified by a polymerase chain reaction (PCR). The ND5 gene was selected as a mitochondrial gene marker as in the previous phylogenetic study on Delias by S.M. (Morinaka et al., 2002). The EF-1α gene was selected as a nuclear marker because it has frequently been used in phylogenetic analyses on various insect lineages (Reed & Sperling, 1999; Vane-Wright et al., 1999; Zakharov et al., 2004). The two genes show different rates of nucleotide substitution, and hence their combination is suitable to infer phylogeny with varying ages of divergence (Müller et al., 2010 and references therein). To amplify ND5, we used primer ‘a’ (forward, 5′-CCTGGTTCTGCTTTAGTTCA-3′) and ‘D5’ (reverse, 5′-TAACCTCTATATATYTCCTCT-3′), which we designed based on the sequences of Drosophila yakuba Burla, Carabus dehaanii Chaudoir, Apis mellifera Linnaeus and several lepidopteran species. To amplify EF-1α, we designed the primers ‘C4’ (forward, 5′-CAATGTGGTATCGA-3′) and ‘B3’ (reverse, 5′-TAACCTCTATATATYTCCTCT-3′), based on the sequences of several papilionid butterflies (Reed et al., 1999). When the target region was not amplified as a whole, additional primers (Supporting Information, Table S1) were used to obtain the target region as a group of shorter fragments. The PCR mixture was prepared in a volume of 50 µL, using the KOD FX system (Toyobo Life Science Department, Osaka, Japan). Amplification was performed with 35 cycles of denaturation at 98 °C for 10 s, annealing at 47–51 °C for 30 s and extension at 68 °C for 1 min. The amplification product was directly sequenced by Macrogen Japan Corp., with the dye terminator cycle sequencing method using primers for PCR amplification.

Nucleotide sequences were aligned using CLUSTAL X with the default setting (Thompson et al., 1994). The best model to explain nucleotide substitution for each of the ND5 and EF-1α data sets was examined using Kakusan3 (Tanabe, 2007) with the number of gamma
Table 1. Taxa used in the present study with collection localities and accession numbers

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<td><em>Delias carstensziana</em></td>
<td></td>
<td>Habbema, West Papua, Indonesia</td>
<td>SZM213</td>
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<tr>
<td><em>Delias toxopei</em></td>
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<td>SZM217</td>
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</table>
which corresponds to the range of the mean ± 2SE in the prior distribution used in Müller et al. (2012). After 50 million generations of the MCMC chains with samples of every 1000 generations run in BEAST, the initial 5 million generations were discarded as burn-in, and samples were summarized in the maximum clade credibility tree using TreeAnnotator v1.8.4 (Drummond et al., 2016) with the ‘common ancestor’ node height option (Heled & Bouckaert, 2013) in effect. The maximum clade credibility tree was visualized using FigTree v1.4.0 (Rambaut, 2012). Analyses were run twice to check for convergence in the topology.

We used R v3.3.2 with the R package BioGeoBEARS (Maztke, 2013a, b, 2014) to infer the possible ancestral ranges of the D. hyparete group on the phylogenetic tree. The biogeographical model of DEC+J was used. BioGeoBEARS was implemented with the BEAST maximum credibility tree from which outgroups were pruned. The geographical range of the D. hyparete group was divided into six areas (Fig. 1; also refer to the present results). These areas are Asia (the Oriental region), the Australian region including Australia, New Guinea and neighbouring islands, Sulawesi (Wallacea), North Maluku (Wallacea), South Maluku (Wallacea) and Lesser Sunda (Wallacea). The maximum number of ancestral areas was set to six. Default settings on dispersal rates between the different areas were used.

RESULTS

No deletions or insertions were detected in any of the 877 or 918 bp of the ND5 or EF-1α gene, respectively. An outline and details of the Bayesian phylogram for the D. hyparete group are shown in Figures 2 and 3, respectively. The D. hyparete group comprised four major clades, as follows. D. sambawana and Delias eileanina Joicey & Talbot formed clade A with a posterior probability of 1.0. Delias salvini Butler was then recognized as a singleton clade with a posterior probability of 1.0 (clade B). Clade C was composed of D. hyparete, D. eucharis, Delias rosenbergi (Snellen van Vollenhoven) and Delias mitisi Staudinger with a posterior probability of 1.0. Clade D included the other

Table 1. Continued

<table>
<thead>
<tr>
<th>Species</th>
<th>Subspecies</th>
<th>Locality</th>
<th>Voucher ID</th>
<th>ND5*</th>
<th>EF1-α*</th>
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</thead>
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<td>Cepora iudith</td>
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</table>

*Accession code in the DDBJ/GenBank/EMBL database.

category set to 4 and default settings for the other parameters. Based on the BIC4 criterion, HKY+G and SYM+G were used as the best models among those available in BEAST v1.8.4 (Drummond & Rambaut, 2007) for ND5 and EF-1α data, respectively. The sequences of the two genes were concatenated using SequenceMatrix (Vaidya et al., 2011).

Bayesian molecular phylogenetic and dating analyses were conducted in BEAST v1.8.4. We unlinkled the substitution and clock models between the two genes, but linked the tree topology model. We used an uncorrelated lognormal relaxed clock model and a birth-death process of speciation for each gene. A UPGMA tree was used as the starting tree. Fifty million generations of the Monte Carlo Markov chains (MCMC) were run, sampling every 1000 generations. Default settings was used for the other parameters. Convergence of the stationary distribution was checked by visually inspecting plots of the posterior estimates using Tracer v1.6.0 (Rambaut et al., 2014). After discarding the initial 5 million generations as burn-in, samples were summarized in the maximum clade credibility tree using TreeAnnotator v1.8.4 (Drummond et al., 2016) with the ‘common ancestor’ node height option (Heled & Bouckaert, 2013) in effect. The maximum clade credibility tree was visualized using FigTree v1.4.0 (Rambaut, 2012). Analyses were run twice to check for convergence in the topology.

After scaling up the branch lengths using Mesquite 3.03 (Maddison & Maddison, 2015), the maximum credibility tree was used as the starting tree in the second round BEAST analysis with the estimation of divergence times. Priors were generally set as in the first round. No reliable internal fossil calibration points were available for the D. hyparete group; therefore, we used a secondary calibration point. Based on previous molecular phylogenetic studies on the family Pieridae (Braby et al. 2006; Braby & Pierce, 2007), Müller et al. (2012) calibrated the molecular phylogeny of Delias using the node between Leucacia and Delias, which are sister genera (Braby et al., 2006). Following Müller et al. (2012), the present study used the node between L. acuta and Delias species as an external calibration point for the D. hyparete group. It consisted of a uniformly distributed prior between 23 and 39 Mya,
D. lara and D. separated the Northern Maluku taxa, i.e. D. poecilea in Wallacea other than Sulawesi. The oldest split in clade bergi and D. mitisi distributed in India and Sri Lanka. Delias rosenbergi and Delias eucharis is widely distributed over continental Asia, but not in Wallacea (except for Lombok). Delias hyparete is closely related to the Flores sample of D. sambawana in Lesser Sunda and Bali. The two species are allopatric. Delias eileenae is endemic to Timor, while D. sambawana is distributed over western Lesser Sunda and Bali. The two species are allopatric. D. sambawana was paraphyletic: D. eileenae was more closely related to the Flores sample of D. sambawana.

Clade C was dominated by Oriental region samples and also included Sulawesi samples. Delias hyparete is widely distributed over continental Asia, but not in Wallacea (except for Lombok). Delias eucharis is distributed in India and Sri Lanka. Delias rosenbergi and D. mitisi are endemic to Sulawesi and its neighbouring islands, and are allopatric to each other. Delias hyparete was paraphyletic because it included both D. rosenbergi and D. mitisi.

Clade D represented the Australian region and Wallacea other than Sulawesi. The oldest split in clade D separated the Northern Maluku taxa, i.e. D. poecilea + D. edela (subclade D-1). The others consisted of three subclades. One subclade included D. lara, D. mysis, D. doylei and D. euphemia, representing New Guinea Island, its neighbouring islands and Australia (subclade D-2). Another subclade was D. ceneus representing Southern Maluku (subclade D-3), which was sister to subclade D-2. The third subclade included D. argenthona, D. periboea, D. timorensis and D. fasciata (subclade D-4), including species from the entirety of Lesser Sunda, Australia, New Guinea and the eastern end of the Oriental region. Delias timorensis was polyphyletic, with the Tanimbar samples basal to the monophyletic group of D. periboea, D. fasciata and D. timorensis from elsewhere (Demar, Babar, Wetar, Sumba, Timor and Moa). Species in clade D, except for D. argenthona, are allopatric to each other.

The chronogram obtained from the BEAST analysis is shown in Figure 4. Clades A–D emerged during a short period: the split between A/C and B/D 8.2 Mya [95% highest posterior density (HPD): 4.5–12.5 Mya], between A and C 7.2 Mya (95% HPD: 3.5–10.7 Mya), and between B and D 7.1 Mya (95% HPD: 3.5–10.7, Mya). In clade A, radiation in Lesser Sunda started 1.5 Mya (95% HPD: 0.60–2.65 Mya). In D. sambawana, the Bali sample split from Lesser Sunda samples 0.19 Mya (95% HPD: 0.01–0.44 Mya). In clade C, after the separation of D. eucharis 4.3 Mya (95% HPD: 2.0–6.8 Mya), D. hyparete spread over Asia from 1.9 Mya (95% HPD: 0.87–3.1 Mya), and then the Sulawesi taxa split 0.90 Mya (95% HPD: 0.31–1.34 Mya). In clade D, the clades for North Maluku, South Maluku and Lesser Sunda samples 0.36 Mya (95% HPD: 0.02–0.45 Mya), and the Kangean sample split 0.08 Mya (95% HPD: 0–0.22 Mya).

The results of ancestral area analyses are shown in Figure 5. Clade A including Asian and Lesser Sunda samples was estimated to have its most recent common ancestor in Lesser Sunda. The range of the most recent common ancestor of clade C including the Oriental region and Sulawesi samples was estimated to be the Oriental region. Regarding clade D, the most recent common ancestor was most likely to have occurred in the Australian region. Subsequently, three dispersal events from the Australian region were inferred: clade D-1 to North Maluku; clade D-3 to South Maluku; and then clade D-4 to Lesser Sunda. Part of clade D-4 reached the Oriental region.

The range of the most recent common ancestor of clades B and D was estimated to be the Australian region. However, the ancestral area of clades A and C was estimated to be Asia or Lesser Sunda. The
ancestral area of the entire *D. hyparete* group was estimated to be Asia, Australian region or Lesser Sunda.

**DISCUSSION**

A molecular phylogenetic analysis of 138 *Delias* species by Müller *et al.* (2012) included 14 species of the *D. hyparete* group. The present study used 18 out of 21 species in the *D. hyparete* group and many subspecies, including six species that have not been used in previous phylogenetic studies of *Delias*. Therefore, the present results markedly increase the taxon sampling of phylogenetic relationships in the *D. hyparete* group, enabling inferences to be made on the diversification process in this group.

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Four major clades were identified in the *D. hyparete* group. They were composed of exclusively different groups of species and closely associated with particular geographical areas. Specifically, clade A represented the Lesser Sunda Islands. Clade B included only *D. salvini*. *D. salvini* and *D. bagoe*, which was not used in the present study, are allopatric sister species in the Bismarck Islands (Yagishita et al., 1993; Müller et al., 2012). Therefore, clade B represents the Bismarck Islands. Clade C included four species from the Oriental region and Sulawesi: *D. hyparete* and *D. eucharis* are principally Oriental species, and *D. rosenbergi* and *D. mitesi* are distributed in Sulawesi and the nearby islands (Yagishita et al., 1993). Four subclades in clade D corresponded to particular geographical regions: clade D-1 represents northern Maluku; clade D-2 the Australian region; clade D-3 southern Maluku; and clade D-4 Lesser Sundas, the eastern end of the Greater Sundas and Australia. Thus, Wallacea was separated into four geographical areas in the phylogeny of the *D. hyparete* group: Sulawesi, Lesser Sundas, northern Maluku and southern Maluku.

A similar separation of Wallacea was previously reported for faunistic diversity. Michaux (2010) reviewed the biogeographical patterns of Wallacean fauna and proposed nine areas of endemism in Wallacea and the Philippines. Wallacea was separated into seven areas: three regions of Sulawesi, northern Maluku, southern Maluku and Timor. The faunistic study of various animal groups and the phylogenetic study of a particular animal lineage led to the recognition of similar areas of endemism, suggesting that a common geological event(s) is involved (cf. Ung et al., 2016; Escalante, 2016).
In the *D. hyparete* group, the divergence age within single areas of endemism in Wallacea was shallow, i.e. <1.2 Mya, the late Pleistocene. During the late Pleistocene, sea level was estimated to be >100 m below the present level for 30% of this epoch (Voris, 2000). According to figures 1 and 2 in the study by Voris (2000),
when the sea level was >100 m below the present level, the islands of North Maluku were connected to each other, and some of the Lesser Sunda islands were connected to each other. This may have contributed to the dispersal of Delias butterflies among the present islands within at least North Maluku and Lesser Sunda, which may have hampered geographical differentiation within areas of endemism. Therefore, the areas of endemism for the D. hyparete group in Wallacea may have finally formed during the late Pleistocene.

Historical dispersal into and out of Wallacea and diversification

The results of the ancestral area analyses conducted in the present study suggest that five events of colonization into Wallacea occurred in the D. hyparete group: colonization into Lesser Sunda in clade A, that into Sulawesi in clade C, that into North Maluku in clade D-1, that into South Maluku in clade D-3 and that into Lesser Sunda in clade D-4. Moreover, evidence for the historical dispersal out of Wallacea was observed, as discussed below.

Clade C in Sulawesi

Delias rosenbergi and D. mitisi are endemic to Sulawesi and the nearby islands. The two species were nested in Asia-dominant clade C and are more closely related to D. hyparete than to D. eucharis. The results of ancestral area and molecular dating analyses suggest that the ancestor of D. mitisi and D. rosenbergi colonized the Sulawesi area from the Oriental region 0.90 Mya. Considering that D. hyparete is paraphyletic to D. mitisi/rosenbergi, the colonizer to the Sulawesi area was the ancestor of D. hyparete or a population of extant D. hyparete. Sulawesi is considered to have taken its present form due to the collision of different islands c. 15–5 Mya (Lohman et al., 2011). Therefore, the colonization of Sulawesi by the ancestor of D. mitisi and D. rosenbergi may have occurred much later after the island collision event. These findings are concordant with the view that Sulawesi's fauna is mostly of an Oriental origin (Lohman et al., 2011). On the other hand, two other butterfly genera distributed in Sulawesi showed different geographical origins: sister relationships between Sulawesi and Timor were found in the phylogeny of Charaxes and Cethosia (Müller et al., 2010). Müller et al. (2010) concluded that the relationship between Sulawesi and Timor was attributable to vicariance rather than historical dispersal.

Clades D-1, D-3 and D-4 in North Maluku, South Maluku and Lesser Sunda

Clade D included six Wallacean species. The results of the ancestral area analyses suggest repeated colonization of Wallacea from the Australian region. Clade D-1 was suggested to have colonized North Maluku 5.0 Mya. Clade D-3 may have colonized South Maluku 3.0 Mya. This is congruent with the geological suggestion that South Maluku emerged above the sea in the past 2 Myr (Lohman et al., 2011). Based on their geological proximity, North Maluku and South Maluku may have been colonized from New Guinea. Clade D-4 was suggested to have colonized Lesser Sunda 1.7 Mya. This is congruent with geological evidence that Timor and other islands in the eastern part of Lesser Sunda are geologically young islands that are c. 2 Myr old (Hall, 2002; Lohman et al., 2011). In clade D-4, D. argenthona was a sister to the Lesser Sunda taxa. This suggests that the ancestor of D. argenthona colonized Lesser Sunda and then subsequently speciated.

Clade A in Lesser Sunda

D. eileenae is endemic to Timor, and D. sambawana is mainly distributed in the western part of Lesser Sunda (Yagishita et al., 1993). The results of the ancestral area analysis suggest that the ancestor of clade A colonized Lesser Sunda independently from clade D; however, these results do not allow us to clarify the geographical origin of clade A because Asia and Lesser Sunda were suggested with similar probabilities. Clades A and C were estimated to have split 7.2 Mya, suggesting that clade A colonized Lesser Sunda before clade D.

Dispersal at the early divergence of the D. hyparete group

The present study found that Asia-, Wallacea- and Australasia-dominant clades (C, A and D, respectively) in the D. hyparete group diverged 7.1–7.2 Mya: this represents early timing in the group's diversification history. This result suggests that the Oriental region, Wallacea and the Australian region were occupied by the D. hyparete group during the late Pleistocene, implying that the ancestors of the D. hyparete group had passed through both Wallace's and Lydekker’s Lines before divergence to clades A–D. This impaction is congruent with a suggestion by Müller et al. (2012) that Wallacea was colonized during early stages of the evolutionary history of the D. hyparete group. Therefore, was the Oriental or Australian region the geographical origin of this species group? The results of ancestral area analyses suggest the Australian region with the highest probability. Müller et al. (2012) previously reconstructed evolutionary changes in the distributional range of the genus Delias: the common ancestor of the D. hyparete group was inferred to have occurred in Australasia or Australasia+Wallacea. Collectively, these findings suggest that the common ancestor of the

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D. hyparete group dispersed from the Australian region toward the Oriental region by way of western Lesser Sunda during the late Pleistocene. This speculation is relevant to two suggestions in geology. Western Lesser Sunda, which was formed by volcanic activity since the Miocene, is considered to be older than eastern Lesser Sunda, which emerged during the Quaternary (Hall, 2002): this may explain clade A butterflies not being distributed in eastern Lesser Sunda. Furthermore, according to figure 2 in Lohman et al. (2011), northern New Guinea (specifically Bird Head) was closer to western Lesser Sunda 5 Mya than it is today: this implies that the geographical origin of the supposed ancestral dispersal toward the Oriental Region in the D. hyparete group may be northern New Guinea.

Dispersal out of Wallacea

When a species is distributed on both sides of Wallace’s or Lydekker’s Line, the direction and timing of trans-border dispersal are of interest. In the D. hyparete group, D. sambawana and D. periboea are distributed in Lesser Sunda and in the eastern part of Greater Sunda. Our results suggest that the ranges of D. sambawana and D. periboea expanded from Lesser Sunda to Greater Sunda. On the chronogram, the Bali sample of D. sambawana split from Lesser Sunda samples 0.19 Mya, and Greater Sunda samples of D. periboea split from Lesser Sunda samples 0.08–0.36 Mya. These results suggest that the two species expanded the range across Wallace’s Line at the end of the middle Pleistocene or the beginning of the late Pleistocene.

Historical dispersal summary

The inferences obtained on the historical dispersal of the D. hyparete group into and out of Wallacea may be summarized as follows (Fig. 6). The most recent common ancestor of the D. hyparete group was presumably distributed in the Australian region. The ancestral species may have dispersed across Lydekker’s and Wallace’s Lines toward the Oriental region by way of the western Lesser Sunda Islands around 7–8 Mya. The colonizers in Lesser Sunda, now recognized as clade A,
have remained there to the present day and have differentiated into two species. The founder population in continental Asia (i.e. clade C) then diverged into *D. hyparete* and *D. eucharis*. The ancestor of *D. hyparete* may have then dispersed back across Wallace’s Line much later and colonized Sulawesi, evolving into present-day *D. rosenbergi* and *D. mitisi*. The ancestral species of the *D. hyparete* group in the Australian region may have dispersed across Lydekker’s Line to South Maluku and North Maluku separately: clades D-1 and D-3 colonized 5.0 and 3.0 Mya, respectively. The former colonizers evolved into *D. edela* and *D. poecilea* in North Maluku, and the latter into *D. ceneus* in South Maluku. Lesser Sunda was subsequently colonized (1.7 Mya) from the Australian region, possibly by the ancestor of *D. argenthalona*, from which *D. timorensis*, *D. periboea* and *D. fasciata* evolved. *D. periboea* and *D. sambawana* subsequently (c. 0.3 Mya) expanded the range across Wallace’s Line to Greater Sunda.

Thus, we infer that the *D. hyparete* group colonized Wallacea repeatedly and from Australian and Oriental regions, and also that dispersals out of Wallacea also occurred repeatedly. This reinforces the recent argument that dispersal into/out of Wallacea was more frequent than classical expectations (Evans et al., 1999; Jönsson et al., 2008, 2010; Balke et al., 2009; Lohman et al., 2011; Tänzer et al., 2013, 2016; Condamine et al., 2015; Toussaint et al., 2015; Toussaint & Balke, 2016). The present study also provides additional evidence for reverse colonization from islands to continents (Bellemain & Ricklefs, 2008; Toussaint et al., 2016). Phylogenetic studies on other animal lineages that diverged in Wallacea may reveal further cases of complex dispersal histories across Wallacea’s and/or Lydekker’s Lines.

The genus *Charaxes* is another butterfly group that is diverse over a wide geographical range of Wallacea. Müller et al. (2010) suggested that Sulawesi was the origin of all *Charaxes* species in the Australian region and Wallacea other than *Charaxes solon*. Namely, a single colonization event into Wallacea was suggested, which is in contrast to the *D. hyparete* group.

**SYSTEMATICS OF THE* D. HYPARETE* GROUP**

Yagishita et al. (1993) classified the *D. hyparete* group into seven subgroups (Table 2). We revised their classification based on the present results and previous findings obtained from molecular phylogenetic analyses.

The wing markings of *D. eileenae* are so unusual in the *D. hyparete* group that its systematic position has remained controversial. Yagishita et al. (1993) treated *D. eileenae* as an independent subgroup, and also stated ‘Yasusuke Nishiyama speculates that *D. eileenae* is the sister species of *D. sambawana*’ (caption to plate 196, which we translated into English). *Delias sambawana* was treated as a member of the *D. periboea* subgroup by Yagishita et al. (1993). Morinaka (2009) suggested a close relationship between *D. sambawana* and *D. eileenae* based on the mitochondrial ND5 sequences of a small number of samples. The present study confirmed the sister relationship between *D. sambawana* and *D. eileenae* using mitochondrial and nuclear genes of a larger sample size.

*Delias timorensis* was regarded as an independent subgroup by Yagishita et al. (1993) due to its unique wing markings in the species group. However, Morinaka (2009) suggested a close relationship between *D. periboea* and *D. timorensis* based on the mitochondrial ND5 sequences of a few samples per species. Similarly, *D. timorensis* appeared to be the closest to *D. periboea* in the phylogenetic tree of mitochondrial and nuclear sequences by Müller et al. (2012). The present study confirmed that *D. timorensis* is close to *D. periboea* and *D. fasciata* based on nuclear and mitochondrial sequences of larger sample sizes.

**Table 2. Classification of the* Delias hyparete* group revised**

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Species</th>
</tr>
</thead>
<tbody>
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<td>Hyparete subgroup</td>
<td><em>D. hyparete</em> (Linnaeus, 1758) [Hy]*</td>
</tr>
<tr>
<td>D. eucharis (Drury, 1773) [Hy]</td>
<td></td>
</tr>
<tr>
<td>D. rosenbergi (Vollenhoven, 1865) [Hy]</td>
<td></td>
</tr>
<tr>
<td>D. mitisi Staudinger, 1894 [Hy]</td>
<td></td>
</tr>
<tr>
<td>Periboea subgroup</td>
<td><em>D. argenthalona</em> (Fabricius, 1793) [Pe]</td>
</tr>
<tr>
<td>D. periboea (Godart, 1819) [Pe]</td>
<td></td>
</tr>
<tr>
<td>D. timorensis (Boisduval, 1836) [Ti]</td>
<td></td>
</tr>
<tr>
<td>D. fasciata Rothschild, 1894 [Pe]</td>
<td></td>
</tr>
<tr>
<td>Schoenbergi subgroup</td>
<td><em>D. schoenbergi</em> Rothschild, 1895 [Pe]</td>
</tr>
<tr>
<td>Poecilea subgroup</td>
<td><em>D. poecilea</em> (Vollenhoven, 1865) [Po]</td>
</tr>
<tr>
<td>D. edela Fruhstorfer, 1910 [Po]</td>
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<tr>
<td>Mysis subgroup</td>
<td><em>D. mysis</em> (Fabricius, 1775) [My]</td>
</tr>
<tr>
<td>D. lara (Boisduval, 1836) [My]</td>
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<td>D. aestiva Butler, 1897 [My]</td>
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<td>D. euphemia Grose-Smith, 1894 [My]</td>
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<td>D. doylei Sanford &amp; Benett, 1955 [My]</td>
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<td>Ceneus subgroup</td>
<td><em>D. ceneus</em> (Linnaeus, 1758) [Po]</td>
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<tr>
<td>Sambawana subgroup</td>
<td><em>D. sambawana</em> Rothschild, 1894 [Pe]</td>
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<td>D. eileenae Joicey &amp; Talbot, 1926 [Ei]</td>
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<td>Bagoe subgroup</td>
<td><em>D. bagoe</em> (Boisduval, 1832) [Ba]</td>
</tr>
<tr>
<td>D. salvini Butler, 1882 [Ba]</td>
<td></td>
</tr>
</tbody>
</table>

*The square brackets show the subgroup classification by Yagishita et al. (1993): Hy, hyparete subgroup; Pe, periboea subgroup; Ei, eileenae subgroup; Po, poecilea subgroup; Ce, ceneus subgroup; Ti, timorensis subgroup; My, mysis subgroup; Ba, bagoe subgroup. The names *D. lara* and *D. aestiva* were not used, but were included in *D. mysis* in Yagishita et al. (1993).*
Based on phylogenetic trees in the present and previous (Müller et al., 2012) studies, we propose eight subgroups of species, as shown in Table 2: D. timorensis is moved to the D. periboea subgroup; D. schoenbergeri is raised to an independent subgroup, based on the findings of Müller et al. (2012); D. ceneus is raised to an independent subgroup, based on the results of the present study and previous findings by Müller et al. (2012); the D. sambawana subgroup is established, and D. eileenae is moved to this subgroup.

In addition, the present phylogenetic analysis showed non-monophyletic status for four species: D. sambawana, D. hyparete, D. mysis and D. timorensis. Delias sambawana was paraphyletic because D. eileenae was included within it in clade A. Similarly, D. hyparete was paraphyletic because D. rosenbergi and D. m. mitisi were included within it in clade C. One explanation for these cases of paraphyly is that D. eileenae and D. rosenbergi/mitisi were recently established from D. sambawana and D. hyparete, respectively, by peripheral speciation events. Another interpretation is that D. eileenae and D. rosenbergi/mitisi are not valid species but geographical races of D. sambawana and D. hyparete, respectively. We argue that the former hypothesis is more reasonable given that the wing markings of D. eileenae and D. sambawana, and those of D. rosenbergi/mitisi and D. hyparete are so different and the species status of D. eileenae and D. rosenbergi/mitisi has not yet been challenged (Supporting Information, Fig. S1).

More interestingly, D. mysis and D. timorensis were each polyphyletic in clade D. D. mysis was polyphyletic because the two subspecies (D. m. mysis and D. m. aruenensis) were included in different subclades, with D. m. aruenensis sister to D. euphemia. D. timorensis was polyphyletic because the Tanimbar samples (D. t. gardineri) were phylogenetically separated from the others. The subspecies D. t. gardineri has a smaller body/wing than the other subspecies of D. timorensis (Yagishita et al., 1993). It is possible that D. m. aruenensis and D. t. gardineri represent distinct species that differentiated slightly in external morphology from the other subspecies of D. mysis and D. timorensis, respectively. This hypothesis needs to be investigated by more closely studying the molecular phylogeny of D. mysis and D. timorensis using more samples and by critically examining external morphology including the male genitalia.

ACKNOWLEDGEMENTS

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

**Table S1.** A list of additional primers used for PCR amplification and its direct sequencing reaction.

**Figure S1.** The diversity of markings on the underside of wings in the *D. hyparete* group. (a) *D. hyparete*, (b) *D. rosenbergi*, (c) *D. argenthona*, (d) *D. periboea*, (e) *D. fasciata*, (f) *D. timorensis*, (g) *D. sambawana*, (h) *D. eileenae*.  

Supplementary Table 1. A list of additional primers used for PCR amplification and its direct sequencing reaction.

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Supplementary Figure 1.

The diversity of markings on the underside of wings in the *D. hyparete* group. (a) *D. hyparete*, (b) *D. rosenbergi*, (c) *D. argenthona*, (d) *D. periboea*, (e) *D. fasciata*, (f) *D. timorensis*, (g) *D. sambawana*, (h) *D. eileenae*. 