A DIVERGENCE TIME ANALYSIS OF BUTTERFLIES IN THE PARADIGM OF THE PLEISTOCENE REFUGIA HYPOTHESIS

By

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ABSTRACT

Several hypotheses have been proposed to explain the diversity and distribution of organisms. A leading hypothesis for biodiversity in the Neotropics, the Pleistocene Refugia Hypothesis (PRH), suggests that a series of climatic oscillations during the Pleistocene Epoch (2.6 mya – 12 kya) produced glacial cycles that periodically isolated plant communities into “islands”. These islands of suitable habitat surrounded by areas of relatively inhospitable habitat became refugia for organisms that required those specific plant communities, and speciation occurred when populations were isolated.

I examined whether or not diversity of extant species of butterflies can be explained by the PRH. To do so, I identified pairs of sister species from previously published phylogenies, and used a molecular clock, based on the mitochondrial DNA CO1 sequence, to infer divergence times for each sister pair. Most species (83%) I examined diverged during the Pleistocene; thus, my data complement a growing body of evidence in support of the PRH.

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ABBREVIATIONS

PRH = Pleistocene Refugia Hypothesis
mya = million years ago
kya = thousand years ago

CHAPTER I: INTRODUCTION

The origins of the current biodiversity in various tropical zones have long been a contested topic of study among systematists, ecologists, biogeographers, phylogeneticists, environmental scientists, and geologists (Haffer, 1969, 1997, 2008; Garzón-Orduña et al., 2014). Studies on the origins of regional biota provide an understanding of how past environmental changes affected biodiversity and, potentially, can be used to predict how current and future environmental changes will affect it; consequently, these studies are an asset to the field of conservation biology (Bennett et al., 2018). One of the more contentious issues is whether or not climatic fluctuations during the Pleistocene generated the present-day tropical diversity, particularly in South America. Some authorities suggest the climatic fluctuations resulted in the formation of restricted habitats that isolated populations, which eventually resulted in allopatric speciation (Pleistocene Refugium Hypothesis (PRH); Prance, 1973, 1982; Colinvaux et al., 2001). The PRH is supported by divergence times of species from various lineages that can be traced to the Pleistocene. For example, Garzón-Orduña et al. (2014) showed that ages of divergence of Neotropical butterflies are consistent with Pleistocene origins. However, Pleistocene climatic fluctuations were a global phenomenon, and it is currently unknown if times of divergence of extant species of butterflies from Afrotropical and Indomalayan regions are also consistent with climatic changes that occurred during the Pleistocene. The primary purpose of my study is to determine if sister species of butterflies inhabiting Africa and Asia diverged during the Pleistocene and, therefore, can also be used in support of the PRH.

Lepidoptera (butterflies, moths, and skippers) is one of the most species-rich groups of arthropods, second only to Coleoptera (beetles) in number of described genera and species (Bouchard, et al., 2017). Because of their presence in tropical rainforests, the availability of a large number of specimens in collections throughout the world, and the wealth of genetic data acquired from them, Lepidoptera are used often to assess the effects of geological and ecological factors on biodiversity. For example, lepidopterans are one of the most studied groups of tropical insects (De-Silva et al., 2017), and frequently are the subjects of research evaluating competing hypotheses of tropical biodiversity (De Jong, et al., 2011; Garzón-Orduña et al., 2014, 2015; De-Silva et al., 2017). Estimates of divergence times of extant species of sister pairs of butterflies allow us to determine (albeit indirectly) whether or not extant lepidopterans are the result of speciation events during Pleistocene climatic oscillations, which allows us to assess the support for the theory of forest fragmentation explained as the PRH.

CHAPTER II: METHODS

To examine the time of divergence of species pairs of tropical butterflies, I identified species pairs from 48 genera from the Afrotropics, 24 genera from the Neotropics, and 19 genera from the Indomalayan tropics. I identified and selected 316 sister pairs (632 species) of butterflies from these regions. I was not interested in selecting specific lineages of butterflies, but rather, focused on identifying sister pairs from the specific regions. Despite their respective groups not being the focus, the species chosen represent a diverse array of genera as organisms from many different lineages coexist in the same environments. I included as many sister pairs as possible in the dataset in order to have a robust sample size. To estimate the age of pairs of sister species from seven families of butterflies, I inferred divergence times of mtDNA COI sequences extracted from either the literature or sequence databases. To ascertain species pairs from the Neotropics, Afrotropics, and Indomalayan realms, most data were acquired from published phylogenies; however, I constructed a matrix and generated a tree using PAUP* (Swofford, 2000) for a few sister species for which a tree was not published for the genus, but for which pertinent sequences were available in GenBank (https://www.ncbi.nlm.nih.gov). I acquired sequences for each species of a pair from GenBank and aligned them by eye, and I used the pairwise distance function in PAUP* to determine the uncorrected p-distances between the sequences of each pair. To acquire estimates of divergence times, pairwise sequence divergence values were converted to values of time since divergence. The conversion of sequence divergence to time is based on a fixed-rate molecular clock of 2.3% divergence per million years as described by Brower (1994). I chose this clock because of its frequent use in arthropod systematics. I also calculated average divergence times for each genus and geographic region by averaging the divergence times of the sister pairs included in the region or group of interest. Lastly, I used published literature to compile a list of suspected Pleistocene refugia and their locations.

CHAPTER III: RESULTS

Overall, I estimated time since divergence for 316 pairs of sister taxa (632 species), which included 48 genera of Neotropical butterflies (147 sister pairs), 24 genera of Afrotropical butterflies (80 sister pairs), and 19 genera of Indomalayan butterflies (89 sister pairs). For most sister pairs from all three geographic regions, the divergence times dated to the Pleistocene Epoch, (2.6 mya – 12 kya). For example, I estimated that 262 of 316 pairs (82.9%) diverged within the last 2.6 my, and the average age of divergence for all pairs was 1.54my. There were small differences in the percentage of species of butterflies that evolved during the Pleistocene in each geographic region, and time of divergence varied among species from the three different tropical realms. For example, most of the Neotropical butterflies I examined originated during the Pleistocene, with 124 of the 147 pairs (84.4%) diverging within the last 2.6my, on average 1.54my. However, only 60 of 80 pairs (75%) of Afrotropical butterflies I examined originated during the Pleistocene. Furthermore, the average time of divergence of the butterflies from the Afrotropics was 1.74my.

Most Indomalyan butterflies speciated a few hundred thousand years after both the extant Neotropical and extant Afrotropical butterflies, with an average of 1.33my. Of the three regions, Indomalaya had the highest percentage of Pleistocene age butterflies with 87.6% diverging during the Pleistocene. The number of refugia thought to have existed during the Pleistocene was similar among the Neotropics (n = 7), Afrotropics (n = 5), and Indomalayan Realm (n = 6) (Table 1).

I describe below many of the groups from my dataset that highlight the similarity in divergence times of species from each of the three tropical regions. I analyzed divergence times from seven of the nine genera in the species-rich Godyridina subtribe of Ithomini in the Neotropics (Table 2). The average divergence time for the subtribe is 1.15my.

Bicyclus is one of the most species-rich genera in the Afrotropics. All of the sister pairs of the *Bicyclus* genus date to the Pleistocene (Table 3). Additionally, I analyzed twelve sister pairs of *Cymothoe*, another species-rich Afrotropical genus. Ten of the twelve *Cymothoe* pairs date to the Pleistocene (Table 3). I also, looked at the divergence times of several genera that included species existing on the island of Madagascar, which is part of the Afrotropics (*Henotesia* and *Colotis*). Divergence times for the island species have a divergence time similar to genera on mainland Africa (Table 3). Average divergence times for nine sister pairs of the genus *Hypolimnas* in the Tribe Kallimini in Africa also date to the Pleistocene (Table 3).

The largest genus I analyzed in the Indomalayan realm was *Delias*. I looked at 49 sister pairs from this genus. The average divergence time for the *Delias* is well within the time frame of the Pleistocene (Table 4). Additionally, I analyzed two of the 12 genera of swallowtails in the Troidini tribe, from the Indomalayan Archipelago (*Troides*, and *Ornithoptera*, Table 4). The average divergence time for the Troidini tribe is 0.74my, an estimate well within the Pleistocene. I looked at average divergence times of eight pairs of arhopaline (Lycaenidae) sister taxa from Indomalaya as well (Table 4). The average divergence time the arhopaline butteflies is 1.82my.

CHAPTER IV: DISCUSSION

Flora affects Phytophagous Fauna

There is a noteworthy connection between hostplant relationships and the PRH (Silva-Brandão, & Solferini, 2007). Presumably, climatic oscillations affected the community composition and distribution of plant species (Prance, 1973, 1982), such that forests were reduced in size and isolated from other patches of forest (= forest fragmentation). Distributions of animals dependent on specific plant species or habitat types tracked the changes in plant distribution; consequently, allopatric speciation of animals was possible because of the isolation of forest habitats (Haffer, 1969, 1997, 2008). If the effects of the climatic cycles during the Pleistocene were global, as paleoecological models suggest (Bush & Oliveira, 2006), then fragmentation of forests and changes in plant community composition should have restricted gene flow among populations of plants used as hosts by butterfly larvae in all three tropical realms, not just the Neotropics. Because many species of lepidopterans coevolved with specific host plants (Ehrlich & Raven, 1964; Silva-Brandão, & Solferini, 2007), information on how climatic oscillations affected the Pleistocene flora will contribute to the understanding of demographic history of pleistocene fauna.

Because the PRH assumes that previosly expansive tropical forests became fragmented into relatively smaller patches, the history of flora expansions and contractions have been explored in conjunction with faunal history. Insect and host plant interactions have been studied for some time now (Silva-Brandão & Solferini, 2007). Presumably, compostion of plant communities change during periods of drying climate, and in particular the density of vegetation might decrease (Prance, 1982). For example, during dry periods, humidity and precipitation both decrease, and changes in these climatic variables affect forest stability in the neotropics (Ramírez-Barahona, & Eguiarte, 2013).

There are multitudes of examples of Amazonian flora forming refugia or showing evidence of expansion and contraction (Prance, 1973, 1982). Prance (1982) identified areas of endemism in Amazonia that formed because of drier environments that developed during glacial cycles (Ramírez-Barahona, & Eguiarte, 2013). Four lowland families of woody plants in the Amazon (Caryocaraceae, Chrysobalanaceae, Dichapetalaceae and Lecythidaceae) all show multiple locations of repeated endemism (Prance, 1973). Sixteen refugia for these four families are proposed by Prance (1973) and largely correspond with refugia proposed for birds by Haffer (1969, 1997, 2008). In the Southern Andes, the Chilean *Nothofagus* forest shows evidence of retreating north, closer to the equator (Vuilleumier, 1971). Furthermore, stratigraphical studies have shown that central Amazonia was largely lowland freshwater forest until the Pleistocene, after which time drier climate conditions hindered the ability for large forest to remain continuous and they began to fragment (Nogueira, Silveira, & Guimarães, 2013). This would have resulted in a loss of vegetation during glacial periods that was amassed during interglacial periods.

Changes in vegetation that reportedly occurred in West Africa during the Pleistocene (Sowunmi et al., 1981) also support the PRH. For example, analyses of pollen samples from cores in western Africa indicate multiple abrupt temporal transitions from lowland rainforest species to drier-adapted savanna species (Sowunmi et al., 1981). The simultaneous decrease in pollen from rainforest species and increase in pollen from savanna species indicates a climatic shift from a moist to a dry environment. The absence of pollen from savanna species at certain times, and then a relatively sudden appearance of them, suggests that there were environments that allowed the savanna adapted species to survive, alternating with environments where they could not have been sustained (Sowunmi et al., 1981).

Tribes of Butterflies with Host Plants

The Troidini tribe of swallowtail butterflies (Papilionidae: Papilioninae), commonly known as Birdwings, exemplify species dependent on specific host plants, and they show a repeated pattern of opportunistic host plant use (Silva-Brandão, & Solferini, 2007). Troidines are associated with plants in the genus *Aristolochia* (pipevines). My data on divergence times for genera in the Troidini are temporally consistent with the PRH.

The tribe Arhopalini (Lycaenidae), native to the Indo-Australian region, is another lineage of butterflies with specific host plant associations. Larvae of these butterlies feed primarily on plants in two families: Fabaceae (legumes) and Fagaceae (oaks and beeches) (Megens, Jong, & Fiedler, 2005). The average divergence time of 1.82my for the eight pairs of arhopaline sister taxa from southeast Asia and Singapore adds further support to the hypothesis that biodiversity of the tropics increased during the Pleistocene. Furthermore, the average divergence time of 2.5my for *Taractrocera* (Hesperiidae), another group of highly specialized butterflies (Braby & Zwick, 2015) indicates that sister pairs within this group also evolved during the Pleistocene.

The neotropical heliconiine butterflies have an ongoing relationship, essentially an evolutionary arms race, with *Passiflora* (Passifloraceae), commonly called passion flower. (*Heliconius* butterflies are often called the passion vine butterfly). Species of

Passiflora manufacture cyanogenic glucosides to thwart predation by insects, and Heliconiines co-evolved chemical defenses to the glucosides, which triggers the plants to modify their chemical compounds (Turner, 1981). Furthermore, Heliconiines larvae sequester the glucosides and use them to deter predation by birds and other predators (Pinheiro et al., 2019). The intimate relationship between *Passiflora* and Heliconiines is often cited as an example of cospeciation (Castro et al., 2017). I suggest that the distribution of Heliconiines tracks that of their host plants, which results in isolation of butterfly populations as the host plant species become isolated.

The larva of the neotropical ithomine tribe of butterflies feed on the plant group Solanaceae (nightshades) (De-Silva et al., 2017). Many species of nightshades eaten by Lepidoptera manufacture toxic pyrrolizidine alkaloids to deter predation (Vrieling & Macel, 2002). Nightshades are found throughout the world but are most diverse in Central and South America (De-Silva, et al., 2017). As with *Heliconius* and *Passiflora*, ithomines sequester toxins ingested from their host plants and use it as a defense against predation (Vrieling & Macel, 2002). Isolation of and subsequent speciation of populations of species of Solanaceae host plants resulted in concomitant speciation in dependent species of ithomine butterflies (De-Silva et al., 2017).

Neotropics

Typically, when people think of the Pleistocene, they think of the megafauna that roamed North and South America, or prehistoric flora (Smith, et al., 2015), yet most groups of animals and plants were by then fairly diverse, including many of the extant genera of butterflies (De Jong et. al. 2011). However, my data indicates that speciation occurred during the Pleistocene within many lineages of butterflies within the Neotropics.

Amazonia is full of highly differentiated habitats that offer diverse opportunities for speciation. The central Amazonian basin offered multiple refuge locations (Beven et al., 1984). The Andes Mountains provide a wide array of habitats, which facilitate higher speciation rates than the surrounding areas and have experienced repeated colonization from them as well (Chazot et al., 2016). The Godyridina subtribe of Ithomine butterflies has experienced an increased rate of diversification in the Andes refugium. My dataset includes seven of the nine genera of the Godyridina subtribe of Ithomini. The average divergence time for the subtribe is 1.15my.

The distribution of *Heliconius* butterflies is consistent with both the geographical and temporal expectations of the PRH. According to the literature (Brown et al., 1974), refuge locations for *Heliconius* butterflies coincide with refuge locations for forest birds proposed by (Haffer, 1969). There are parallels to *Heliconius* butterflies in other regions as well. For example, "Afrotropical Acraeini butterflies provide a fascinating potential model system to contrast with the Neotropical Heliconiini" (Timmermans et al., 2016, p. 233). They both display evidence for episodic diversifying selection.

Afrotropics

The nymphalid genus *Bicyclus* is one of the largest groups of butterflies in the Afrotropics (Aduse-Poku et al., 2016a). All pairs of *Bicyclus* sister species have divergence times less than 2.6my, which supports the suggestion by De Jong et al., (2011) that this genus radiated rapidly during the Pleistocene. To quantify genetic and regional variation, De Jong, et al. (2011) examined six widely-separated populations (from Uganda to South Africa) of the Afrotropical butterfly *Bicyclus anynana* and found that those located further south were more divergent from ancestral populations than extant populations towards the equator, suggesting an expansion from equatorial regions further south. They suggest that the distribution of species was restricted to various refugia in equatorial Africa during the Pleistocene.

Cymothoe, a species-rich genus of butterflies that inhabits the tropics, including the tropical forests of Africa, also appears to have diversified because of climatic oscillations during the Pleistocene (Velzen et al., 2013). Divergence times were earlier than 2.6my in only two of the 12 pairs of sister species I examined in this genus, with an average divergence time of about 0.72my.

The diversity of mtDNA of butterflies on the island of Madagascar was fueled by a refuge in central Madagascar (Dausmann & Warnecke, 2016, my data). There were many refugia on the mainland of the African continent (Linares et al., 2009), including the Gulf of Guinea in central Africa (Ntie et al., 2017) and other locations (my compilation). Furthermore, divergence times for some of the butterflies in the genera *Henotesia* and *Colotis* in Madagascar are similar to those of mainland Africa. For example, 15 pairs of sister species in the genus *Colotis* (orange tips; Pieridae) have an average divergence time of 1.75my. *Pareronia* (Pieridae), a mainland genus in the same family with *Colotis* (Nazari et al., 2011), has an average divergence time of 0.21my, roughly the same time as the island species. An example pair from the island genus *Henotesia*, (*H. turbans, and H. sabas*) show a divergence time of 0.80my, coinciding with the Pleistocene. Forest fragmentation during the Pleistocene is suggested as the cause of current diversity of Madagascan *Henotesia* butterflies (Linares et al., 2009). The subtribe Mycalesina, which includes the genus *Henotesia*, also has experienced large radiations in Madagascar, based on Mitochondrial DNA (Torres et. al., 2001). The

Mycalesina satyrine butterflies are represented in three regions: *Heteropsis* in Madagascar, *Telinga* in Asia, and *Brakefieldia* in Africa (Aduse-Poku et al., 2016b). I examined some butterflies that originated in the Afrotropics, including species in the genus *Junonia* (Buckeyes; Tribe: Junoniini; Family Nymphalidae; Wahlberg, Brower, & Nylin, 2005). The average divergence time of 2.05my for the three sister pairs is consistent with a Pleistocene origin. Furthermore, average divergence time of 1.78my for the nine sister pairs genus *Hypolimnas* of (Tribe Kallimini: diadem butterflies (Nymphalidae) is also consistent with Afrotropic diversity increasing during the Pleistocene.

Indomalayan Realm

This geographical region offers an interesting perspective into how the Pleistocene climatic changes affected large groups of islands or archipelagos as opposed to land masses. Many refugia were scattered across the various islands. As stated in Müller and Beheregaray (2010), the Indo-Pacific region's complex geology and tectonic history makes the region unique. Although not a continental land mass, this realm has experienced forest fragmentation. Gathorne-Hardy et al. (2002) indicate that changes in sea level in south-eastern Asia created a significant amount of fragmentation during the Pleistocene. My data on average divergence times of sister species in several genera inhabiting the Indomalayan Realm support the PRH. For example, the average divergence time of 1.7my for the 49 pairs of sister species in the genus *Delias* indicates that diversity of this group occurred primarily during the Pleistocene. This is particualry interesting because *Delias*, one of the largest genera of butterflies, is distributed across the entire range of the Indo-Pacific, and many of the species exhibit local endemism (Müller, Matos-Maraví, & Beheregaray, 2013).

Genera spanning multiple regions exhibit the same patterns in each region

My data show that divergence times are similar among congeneric butterflies sampled from disparate geographical locations. For example, divergence times of nymphalid butterflies in the genus *Charaxes* are similar among clades from central Africa and the Indo-Pacific region, specifically the islands of Wallacea (Müller, Wahlberg, & Beheregaray, 2010). My data include divergence times for 26 species in each region. Of the 52 species analyzed, only two species pairs have divergence times older than the Pleistocene, with an average divergence time of 1.66mya for the 13 species pairs of *Charaxes* in central African and of 1.35mya for the species pairs in Indomalaya.

Although the Junoniini originated in Africa (Wahlberg, Brower, & Nylin, 2005), species included in this clade are represented in all three geographical regions. Diversification times of species in the genus *Junonia* (buckeyes) range from 2.05my for African species, 1.04my for South American species, and 1.92my for Australasian species. Thus, divergence times for species from this genus occurred during the Pleistocene in all three regions.

By comparing families in three geographic realms, I found that among families the species in each region had similar divergence times. For example, in the Nymphalidae, the South American average divergence time is 1.67my representing 43 genera, the African average divergence time is 1.60my representing 12 genera, and the Indomalayan average divergence time is 1.59my representing 12 genera. Similarly, in the Riodinidae, the South American average is 2.20my, and the Indomalayan average is

1.89my. Additionally, in the Papilionidae family, the South American average is 1.78 my, and the Indomalayan average is 0.74my.

My results allow a comparison of divergence times within families amongst the three tropical regions. For example, the family Riodinidae is primarily represented in the Neotropics. “This family is distributed worldwide, but more than 90% of the 1500 species are found in the Neotropics, while the 120 Old World species are concentrated in the Southeast Asian tropics, with minor Afrotropical and Australasian tropical radiations” (Espeland et al., 2015, p. 296). As stated in in Espeland et al. (2015), this family of lepidopterans has representatives in all three of the tropical regions, which allowed within family assessments. For example, the average divergence time for sampled sister pairs in the family Riodinidae across the tropics is 1.89my.

Why is the PRH supported in the Paleotropics and not the Neotropics?

The PRH has been given as an explanation for extant diversity in many publications for the Afrotropics and Indomalayan regions, but it is contested in the Neotropics (Garzon-Orduña et al., 2014, 2015). There are several possible reasons for this. The Neotropics contain a vast and intricate river system that is lacking in each of the two Paleotropic realms. The presence of the complex river system in Amazonia offers an alternative explanation for increased diversity, the River Barrier Hypothesis (Haffer, 2008). River systems within the Afrotropics are not as complex as the Amazonian river system and, the Amazonian rivers are thought to have served as a geographical barrier for allopatric speciation (Naka and Brumfield, 2018). The Andes Mountains are another geological feature in South America that are potentially associated with diversification of species in the region (as opposed to climatic oscillations). “Fjeldsa has argued that the

Andes, after their formation during the Tertiary (65 million to 1.64 million years ago), became the species pump for the entire region" (Knapp & Mallet, 2003, p. 72). I do not doubt that the Andes Mountains fragmented habitat in western Amazonia and are likely responsible for some of the biodiversity in that region; however, the significane of the mountain range's impact on diversification and, hence, biodiversity of the central and eastern Amazonia may be overstaded.

Pleistocene glaciations have been used to explain refugia across the islands of the Indomalayan Archipelago. According to Morley (2018), the PRH is supported in the Indomalaya in contrast to the views in the Neotropics. This may be due to the geography of the region, i.e., the Indomalayan Archipelago is not one large land mass comparable to the Neotropics. Rather, it is a conglomerate of archipelagoes, individual islands large and small, and the southern-most part of Asia. Groups of island chains may be more susceptible to forest fragmentation (Morley, 2018) and, smaller land masses are more sensitive to changing sea levels. Southeast Asia has gone through recurring episodes of inundation and exposure, resulting in shifts from wetter to drier climates (Morley, 2018).

Lack of support for the PRH in the Afrotropics may come from differences in the current state of the Afrotropic landscape compared to that of the Neotropics. For example, Africa displays less forest cover than South America, perhaps because the forest cover in Africa is in the form of belts interspersed among grassland savannas. The two large ones are the Sahel belt and the Sudan belt (Černý et al., 2007). The current fragmented nature of Afrotropical forest compared to the continuous forests of Neotropics may be a reason why the PRH is accepted more in the former. There are also vast savannas in East Africa, such as the Serengeti, which is the type of environment that replaced forest during interglacial periods, and that are not present at the same levels in South America. Madagascar and the various small islands throughout the eastern Indian Ocean are part of the Afrotropics, which add to the inherent fragmentary nature of the region.

Caveats and future directions

It is important to recognize that the inferences made in this work are based on a single gene region (CO1) from the mitochondrial genome. To further test the validity of these inferences, it will be necessary to use multiple mitochondrial genes. Additionally, the present study makes use of mtDNA because of its widespread use to diagnose relatively recent relationships, such as the sister pairs observed here. Genes from the nuclear genome can be used in conjunction with the mtDNA to better reflect the evolutionary history of these organisms. Until that time, this work provides an important first step towards elucidating time frames for diversification of butterflies around the globe.

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Table 1. Locations of areas previously proposed as Pleistocene Refugia in the three tropical regions explored in this study.

Refugia Locations		
Neotropics		
Name of Refuge	Location	Reference
Imerl	Central Amazon Basin	Beven et al., 1984
Rondonia	Central Amazon Basin	Beven et al., 1984
Guiana	Guiana Highlands	Beven et al., 1984
Napo	Andes Mountains	Beven et al., 1984
Inambari	Andes Mountains	Beven et al., 1984
Belem	Northeast of the Brazilian highland plateau	Beven et al., 1984
Falkland Islands	Off the coast of Argentina	Vuilleumier, 1971
Afrotropics		
Upper Guinea forest	Lowland forest of west Africa	Mayr & O'Hara, 1986
West lower Guinea forest	Lowland forest of west Africa	Mayr & O'Hara, 1986
East lower Guinea forest	Lowland forest of west Africa	Mayr & O'Hara, 1986
Gulf of Guinea	Central Africa	Ntie et al., 2017
Central Madagascar	Central Madagascar	Dausmann & Warnecke, 2016
Indomalayan Realm		
Yunnan (China)	Southwestern China	Sakka et al., 2010
Sichuan (China)	Southwestern China	Sakka et al., 2010
Taiwan	Northwestern and Southeastern Taiwan	Oshida et al., 2011
Borneo	Northern and Eastern Borneo	Gathorne-Hardy et al., 2002
Sumatra	Western Sumatra	Gathorne-Hardy et al., 2002
Mentawai Islands	Mentawai Islands	Gathorne-Hardy et al., 2002

Table 2. Divergence times of species pairs of Neotropical butterflies estimated using mtDNA (CO1) and a molecular clock rate of 2.3% divergence per my in genera examined.

Neotropical Species Pairs			
Genera	Family: Subfamily	Divergence Time in Millions of Years	Reference
Pseudoscada	(Nymphalidae: Danainae)	1.993478my	Tree created in PAUP
Hypomenitis	(Nymphalidae: Danainae)	1.005my	Chazot et al., 2016
Heterosais	(Nymphalidae: Danainae)	1.652609my	Chazot et al., 2016
Pachacutia	(Nymphalidae: Danainae)	1.323913my	Chazot et al., 2016
Brevioleria	(Nymphalidae: Danainae)	0.808696my	Chazot et al., 2016
Hypoleria	(Nymphalidae: Danainae)	0.915652my	Chazot et al., 2016
Godyris	(Nymphalidae: Danainae)	0.458551my	Chazot et al., 2016
Veladyris	(Nymphalidae: Danainae)	1.08087my	Chazot et al., 2016
Velamysta	(Nymphalidae: Danainae)	1.143478my	Chazot et al., 2016
Pteronymia	(Nymphalidae: Danainae)	1.199006my	De-Silva et al., 2017
Heraclides	(Palpilionidae: Palpilioninae)	0.716my	Grishin et al., 2014
Pterourus	(Palpilionidae: Palpilioninae)	2.844348my	Garzón-Orduña et al., 2014
Hamadryas	(Nymphalidae: Biblidinae)	1.521739my	Garzón-Orduña et al., 2014
Adelpha	(Nymphalidae: Limenitidinae)	1.502415my	Garzón-Orduña et al., 2014
Hyposcada	(Nymphalidae: Ithomiinae)	1.478261my	Garzón-Orduña et al., 2014
Ithomia	(Nymphalidae: Ithomiinae)	0.930435my	Garzón-Orduña et al., 2014

Morpho	(Nymphalidae: Morphinae)	2.298137my	Garzón-Orduña et al., 2014
Janatella	(Nymphalidae: Nymphalinae)	1.0my	Garzón-Orduña et al., 2014
Eresia	(Nymphalidae: Nymphalinae)	1.728261my	Garzón-Orduña et al., 2014
Eueides	(Nymphalidae: Heliconinae)	2.362319my	Garzón-Orduña et al., 2014
Heliconius	(Nymphalidae: Heliconinae)	1.887681my	Garzón-Orduña et al., 2014
Lymanopoda	(Nymphalidae: Satyrinae)	1.889328my	Garzón-Orduña et al., 2014
Hypanartia	(Nymphalidae: Nymphalinae)	1.434783my	Garzón-Orduña et al., 2014
Junonia	(Nymphalidae: Nymphalinae)	1.0434478my	Garzón-Orduña et al., 2014
Hamabryas	(Nymphalidae: Biblidinae)	1.934783my	Garzón-Orduña et al., 2014
Blepolenis	(Nymphalidae: Satyrinae)	0.434783my	Garzón-Orduña et al., 2014
Dasyopthal	(Nymphalidae: Satyrinae)	2.391304my	Garzón-Orduña et al., 2014
Baeotus	(Nymphalidae: Nymphalinae)	3.826087my	Garzón-Orduña et al., 2014
Parataygetis	(Nymphalidae: Satyrinae)	2.173913my	Garzón-Orduña et al., 2014
Forsterinaria	(Nymphalidae: Satyrinae)	1.591304my	Garzón-Orduña et al., 2014
Oleria	(Nymphalidae: Ithomiinae)	1.967391my	Garzón-Orduña et al., 2014
Taygetis	(Nymphalidae: Satyrinae)	1.971014my	Garzón-Orduña et al., 2014
Napeogenes	(Nymphalidae: Ithomiinae)	1.681159my	Garzón-Orduña et al., 2014
Perisama	(Nymphalidae: Biblidinae)	0.513043my	Garzón-Orduña et al., 2014
Hypothyris	(Nymphalidae: Ithomiinae)	1.173913my	Garzón-Orduña et al., 2014
Hyaliris	(Nymphalidae: Ithomiinae)	0.565217my	Garzón-Orduña et al., 2014
Forbestra	(Nymphalidae: Ithomiinae)	0.086957my	Garzón-Orduña et al., 2014
Consul	(Nymphalidae: Charaxinae)	0.434783my	Garzón-Orduña et al., 2014

Oressinoma	(Nymphalidae: Satyrinae)	2.826087my	Garzón-Orduña et al., 2014
Gnathotriche	(Nymphalidae: Nymphalinae)	1.532332my	Garzón-Orduña et al., 2014

Table 3. Divergence times of species pairs of Aftrotropical butterflies estimated using mtDNA (CO1) and a molecular clock rate of 2.3% divergence per my in genera examined.

Afrotropical Species Pairs			
Genera	Family: Subfamily	Divergence Time in Millions of Years	Reference
Henotesia	(Nymphalidae: Satyrinae)	3.532174my	Torres et al., 2001
Bicyclus	(Nymphalidae: Satyrinae)	0.816348my	Aduse-Poku et al., 2016a
Nepheronia	(Pieridae: Pierinae)	4.342609my	Nazari et al., 2011
Colotis	(Pieridae: Pierinae)	1.74587my	Nazari et al., 2011
Junonia	(Nymphalidae: Nymphalinae)	2.035797my	Wahlberg et al., 2005
Cymothoe	(Nymphalidae: Limenitidinae)	0.721014my	Velzen et al., 2013
Charaxes	(Nymphalidae: Charaxinae)	1.66198my	Aduse-Poku et al., 2009
Euphaedram	(Nymphalidae: Limenitidinae)	0.765478my	Dhungel & Wahlberg, 2018
Hypolimnas	(Nymphalidae: Nymphalinae)	1.775652my	Sahoo et al., 2018
Bermatistes	Nymphalidae	2.938043my	Timmermans et al., 2016 Silva-Brandão et al., 2008
Euxanthe	(Nymphalidae: Charaxinae)	1.471087my	Dhungel & Wahlberg, 2018
Precis	(Nymphalidae: Nymphalinae)	4.155507my	Wahlberg et al., 2005
Antanartia	(Nymphalidae: Nymphalinae)	0.7my	Wahlberg et al., 2005
Lipalphnaeus	(Lycaenidae: Theclinae)	1.741743my	Boyle et al., 2014

Table 4. Divergence times of species pairs of Indomalayan butterflies estimated using mtDNA (CO1) and a molecular clock rate of 2.3% divergence per my in genera examined.

Indomalayan Species Pairs			
Genera	Family	Divergence Time in Millions of Years	Reference
Omithoptera	(Papilionidae: Papilioninae)	0.85my	Condamine et al., 2015
Troides	(Papilionidae: Papilioninae)	0.631087my	Condamine et al., 2015
Charaxes	(Nymphalidae: Charaxinae)	1.352451my	Aduse-Poku et al., 2009 Müller et al., 2010
Polyura	(Nymphalidae: Charaxinae)	2.71087my	Aduse-Poku et al., 2009
Pareronia	(Pieridae: Pierinae)	0.205217my	Nazari et al., 2011
Colotis	(Pieridae: Pierinae)	3.237826my	Nazari et al., 2011
Vanessa	(Nymphalidae: Nymphalinae)	3.249565my	Wahlberg et al., 2005
Junonia	(Nymphalidae: Nymphalinae)	1.93913my	Wahlberg et al., 2005
Delias	(Pieridae: Pierinae)	1.174571my	Morinaka et al., 2017 Müller et al., 2013
Arhopala	(Lycaenidae: Theclinae)	1.81538my	Megens et al., 2005
Telicota	(Hesperiidae: Hesperiinae)	1.189565my	Braby & Zwick, 2015
Ocybadistes	(Hesperiidae: Hesperiinae)	2.444783my	Braby & Zwick, 2015
Taractrocera	(Hesperiidae: Hesperiinae)	2.51087my	Braby & Zwick, 2015
Praetaxila	(Riodinidae: Nemeobiinae)	1.888696my	Espeland et. al., 2015
Ypthima	(Nymphalidae: Satyrinae)	0.864348my	Osozawa et al., 2017

Pithecops	(Lycaenidae: Polyommatinae)	4.330435my	Vila et al., 2011
Euploea	(Nymphalidae: Danainae)	0.947826my	Tree created in PAUP
Danaus	(Nymphalidae: Danainae)	0.804348my	Smith et al., 2005
Cethosia	(Nymphalidae: Heliconiinae)	0.250522my	Müller & Beheregaray, 2010
Parantica	(Nymphalidae: Danainae)	1.435797my	Tree created in PAUP

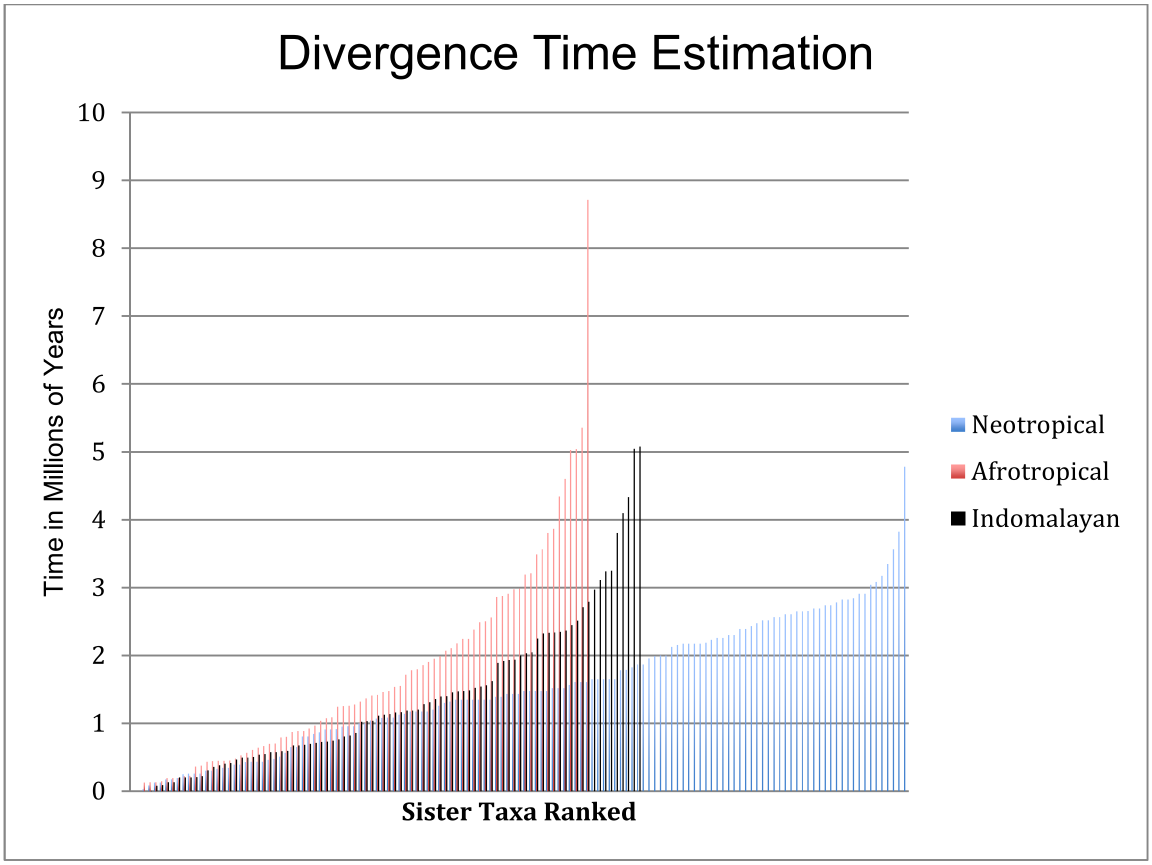

Figure 1. Divergence times of exemplar sister species of butterflies for each of three tropical realms. Each bar represents a pair of sister taxa, ranked in order from the shortest divergence time to the longest across the x-axis. The Pleistocene Period goes from 2.6 myr to 12 kya.

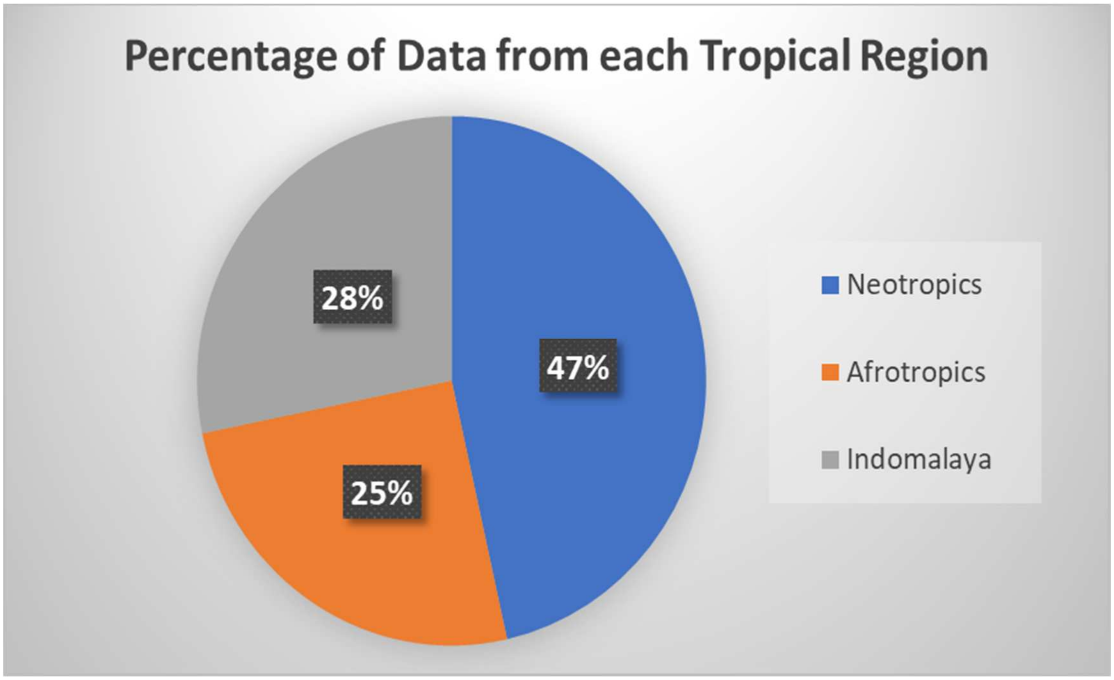

Figure 2. Percentage of sister pairs of butterflies from the Neotropics, Afrotropics, and Indomalyan Realm.