Effect of Climatic Conditions and Land Cover on Genetic Structure and Diversity of *Eunica tatila* (Lepidoptera) in the Yucatan Peninsula, Mexico

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Abstract: Fragmentation is the third cause of the biodiversity declination. Population genetic studies using Lepidoptera as the model species in the context of loss of habitat are scarce, particularly for tropical areas. We chose a widespread butterfly from Mexico as the model species to explore how changes of habitat characteristics (undisturbed forest, anthropogenic disturbances, and coastal areas), and climatic conditions affect genetic diversity and population structure. The Nymphalidae *Eunica tatila* is a common species in the Yucatan Peninsula considered to be a bio-indicator of undisturbed tropical forest, with migratory potential and a possible sex-biased dispersal. We genotyped 323 individuals collected in eight undisturbed areas, using four Inter Simple Sequence Repeats primers. Results show a high genetic diversity and no population structure. Temperature and shrub density present a positive and significant relationship with polymorphism values. Furthermore, our results show the positive effect of surrounding forest habitat on genetic diversity, confirming that *E. tatila* is a bio-indicator of undisturbed tropical forest. We found evidence of sex-biased dispersal. This paper represents one of the few studies on population genetics of tropical butterfly in a fragmented landscape and is, therefore, an important step in understanding the impact of habitat fragmentation on the risk of a butterflies' decline.

Keywords: butterfly; fragmentation; tropical; ISSR; sex-biased dispersal

1. Introduction

The impact of habitat fragmentation on biology and biodiversity is one of the most important issues of this century. Fragmentation results in the reduction of the total habitat by transforming large and continuous areas of habitat into smaller and isolated fragments within a matrix, defined as zones of now-inhospitable terrain between patches [1,2] However, the way that the species perceived the patch and the matrix differed between them, making an important difference in regard to the level of isolation [3]. Fragmentation may be caused by natural processes (e.g., fire and wind, etc.) or human activities (e.g., agriculture and urbanization, etc.) [1], and often results in a reduction in population size, thus, decreasing genetic diversity and connectivity [2]. Connectivity in a fragmented habitat can

be considered as structural if only landscape structure is considered, or functional when movements of individuals (or genes) into the landscape are addressed [4].

Short lifecycles and variability for their resilience capacities make insects good bio-indicators [5]. Many studies have demonstrated the advantages of Lepidoptera as bio-indicators in tropical [6–9] or temperate [10,11] ecosystems. For the Yucatan Peninsula, Pozo (2006) [12] endorsed nine butterfly species as environmental indicators of undisturbed tropical forest and seven for disturbed tropical forest.

Dispersal capacity of species, movement of individuals leading to gene flow among local populations, is a key factor for the permanence of species. Over the last several decades, many studies have shown the complexity of the causes involved in the dispersal process (see reference [13] for a review). Many ecological, phenotypical, environmental, and social conditions [13], were investigated to understand butterfly dispersal, but one important inherent factor is the sex of individuals. Ducatez et al. (2013) [14] demonstrated that habitat connectivity in a fragmented landscape is sex-dependent, with male flight capacities increasing with decreasing habitat connectivity, while females present an inverse pattern. Many butterfly species have high vagility, and their ability to fly may be considered as a contributing factor to genetic homogenization [15]. Consequently, a high level of gene flow between populations of these species was expected. However, recent studies have contradicted this assertion, demonstrating that natural processes of fragmentation can strongly affect population structure. Machkour-M'Rabet et al. (2014) [16] presented evidence that a natural barrier, such as a major river, can result in more differences between nearby populations of *Baronia brevicornis* Salvin, 1893 (Lepidoptera: Papilionidae) than large geographic distances within a homogeneous landscape. Leidner and Haddad (2010) [17] provided evidence that natural barriers affect the dispersal of the coastal endemic crystal skipper butterfly (*Atrytonopsis quinteri* Burns, 2015; Lepidoptera: Hesperiidae) to a larger degree than urbanization. Anthropogenic fragmentation, such as agriculture generating a matrix of non-habitat (no native vegetation), produces a loss of genetic diversity as shown for the speckled wood butterfly (*Pararge aegeria* Linnaeus, 1758; Lepidoptera: Nymphalidae) in Belgium, where genetic diversity was significantly higher in continuous woodlands than in fragmented and isolated woodlands [18]. Williams et al. (2003) [19] working on *Speyeria idalia* Drury, 1773 (Lepidoptera: Nymphalidae) demonstrated that populations present an increase in genetic differentiation while the connectivity among them decreases. Álvarez et al. (2005) [20] emphasize the importance of butterfly dispersal, showing that species with a lower dispersal capacity present less gene flow; therefore, natural and anthropogenic barriers represent an important barrier for such species. Butterfly abundance and population size are, in part, related to environmental and climatic conditions (humidity, temperature, rainfall, plant density, patch size, and nectar abundance among others) [21,22] and could determine the level of genetic diversity of a population [23].

The Yucatan Peninsula is a biodiversity hotspot and is considered as a priority area for conservation [24]. The Yucatan Peninsula, situated in southeastern Mexico, includes three Mexican states: Quintana Roo, Campeche, and Yucatan. The region presents annual mean temperatures that range from 18 °C to 28 °C and annual rainfall between 500 mm to 1800 mm. Its vegetation is typical of a tropical region, with evergreen, semi-evergreen, deciduous, and semi-deciduous forest, as well as lowland floodplain and wetland [25,26]. The Yucatan Peninsula has a simple topography with lack of evident geographical barriers and low habitat heterogeneity [27]. Due to its position, the Yucatan Peninsula is hit by hurricanes on the east coast with a gradient of humidity that determines extensive areas, from northeast to southwest, with deciduous or semi-deciduous forests [27,28]. The Yucatan Peninsula has several protected areas, some are official and others under private control. The predominant economic activity varies from the north to the south of the Peninsula: In the north, tourism dominates while the south depends more on agriculture and livestock activities. The dry tropical forests of the Yucatan Peninsula have been exploited since the time of the Mayas; one of the main theories of the collapse of the Mayan civilization is the deteriorating environment that was caused by increased population density [29]. A second overexploitation of resources commenced in

1880 with the initiation of timber exploitation [30]. More recently, the transformation of forest to an agricultural landscape is the main cause of deforestation in the Yucatan Peninsula, as well as the high development of human activities and infrastructure, particularly in Quintana Roo state [30]. In this context, the Yucatan Peninsula is a perfect example of how fragmentation, principally because of human activities, may affect species, their genetic diversity, and the connectivity between populations. Then, butterflies from the Yucatan Peninsula may provide an excellent model to understand the effect of the fragmentation on the genetic diversity of tropical butterflies.

According to Pozo et al. (2011) [31], 550 species of butterflies have been recorded and widely studied in the Yucatan Peninsula, attaining a significant amount of knowledge regarding their taxonomy, ecology, and distribution [21,31–36]. In the Yucatan Peninsula, one of the most abundant butterfly species in undisturbed areas is *Eunica tatila* Herrich-Schäffer, 1855 (Lepidoptera: Nymphalidae). *Eunica tatila* is one of the most common and widespread species in the *Eunica* genus. It is native to the Neotropics, and presents three subspecies (see reference [37] for details; Figure S1). However, our recent study [37] based on morphological and morphometrical analysis, highlighted the possibility of mixed individuals between two subspecies, *E. tatila* and *E. tatila tatilista*, the latter a subspecies from southern Florida and the West Indies. Therefore, we use the name of the specie, *E. tatila*, until this issue is resolved. As *Eunica tatila* has been identified as a bio-indicator species of undisturbed areas in the Yucatan Peninsula [34], as well as having a widespread distribution and high population density in the Yucatan Peninsula, this species represents a good candidate to understand the effect of fragmentation.

Our goal is to evaluate the effect of land cover and climatic conditions on the genetic diversity and population structure of *E. tatila*. This study will attempt to answer the following research questions: (i) Has genetic diversity been affected by climatic conditions and/or land cover? (ii) Has the distance between preserved patches affected the connectivity of *E. tatila* populations? (iii) Does *E. tatila* present a metapopulation in the Yucatan Peninsula? and, finally, (iv) Does *E. tatila* present signs of sex-biased dispersal?

2. Materials and Methods

2.1. Study Sites and Butterfly Samples

All individual butterflies were collected from eight undisturbed (>20 years without human perturbations) protected areas in the three states of the Yucatan Peninsula (Figure 1). One site was located in Campeche state: (1) "Reserva de la Biosfera de Calakmul" (CK; 18°31′ N–89°49′ W) which has federal protection and a total area of 723,185 ha. Two sites were located in the Yucatan state: (2) "El Zapotal" (ZP; 21°23′ N–87°40′ W), which is a private reserve and covers a total area of 2358 ha, (3) "Reserva Biocultural Kaxil Kiuic" (KU; 20°05′ N–89°33′ W) which is also privately protected, with a total area of 1650 ha. In the state of Quintana Roo we collected at five different sites: (4) "Kohunlich" (KH; 18°25′ N–88°47′ W), an archeological site under federal protection with an area of 8.5 ha, (5) "Miguel Hidalgo" (MH; 18°54′ N–88°21′ W) a village near the town of Bacalar which receives community protection (an area of 2262 ha), (6) "Parque Nacional Arrecifes de Xcalak" (XC; 18°14′ N–87°52′ W) with federal protection and an extension of 4543 ha of terrestrial protection, (7) "Reserva de la Biosfera Sian Ka'an" (SK; 19°48′ N–87°38′ W), a federal protected area with 375,012 ha of terrestrial protection, and finally, (8) "Jardín Botánico Dr. Alfredo Barrera Marín" (JB; 20°50′ N–86°54′ W) protected by the "Consejo Nacional de Ciencia y Tecnologia (CONACYT)" since 1982, covering an area of 65 ha.
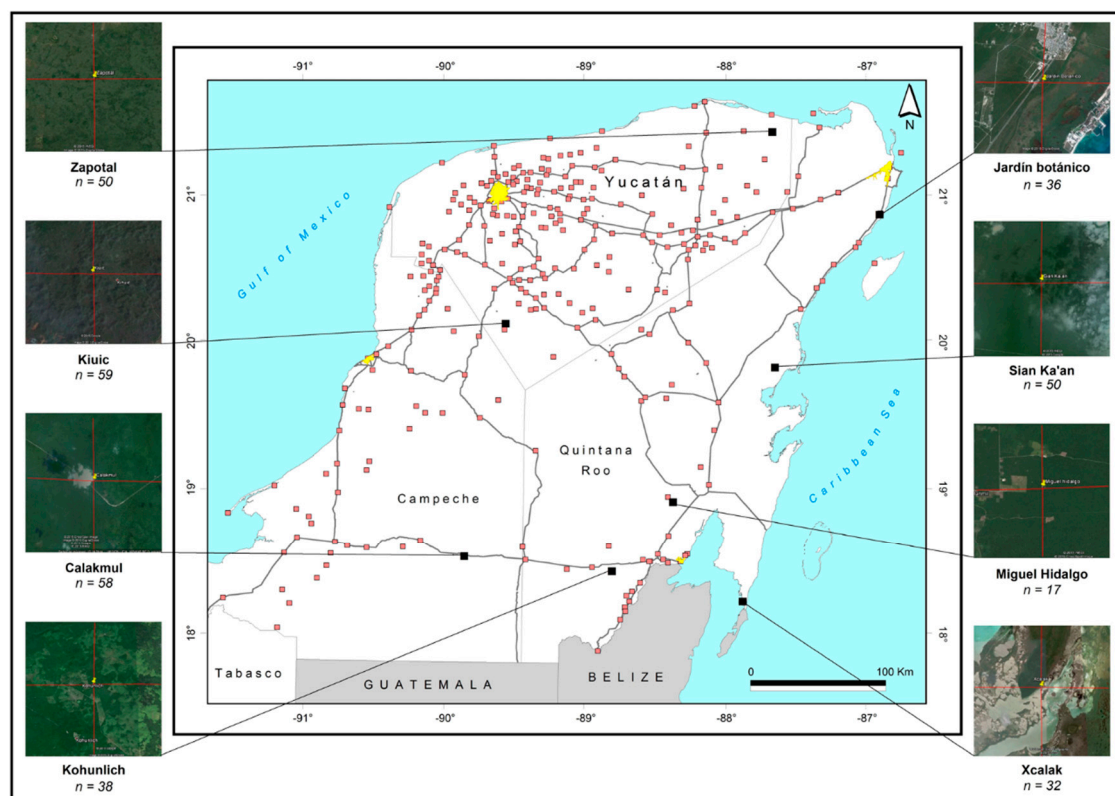
Figure 1. Geographical location of the eight sites in the Yucatan Peninsula (Mexico) where *Eunica tatila* was collected. For each locality, we present the 16 km^2 square used to characterize the land cover of each area (refer to material and methods for more information). Number of collected individuals (n), principal cities (Chetumal, Cancun, Merida, Campeche) (yellow squares), and cities with more than 1000 inhabitants (red squares).

During the sampling (2013), some variables were registered: (1) Climatic conditions (rainfall, humidity, and temperature; annual mean over data from the collection year) were measured using data from the national meteorological stations (http://smn.cna.gob.mx/es/emas) nearest to each site, (2) ecological conditions with tree density (10 quadrants of 400 m^2), shrub density (10 quadrants of 25 m^2), and herbaceous plant density (10 quadrants of 1 m^2) [38], and, finally, (3) patch area (taken from the conservation management plan of each area). Furthermore, landscape constitution was evaluated as follows: A 16 km^2 square around each sample point was examined (2 km from a central point to each cardinal side using GOOGLE EARTH; Figure 1). Within these squares, a 100 m × 100 m grid pattern was drawn, and we recorded the proportion of forest area, coastal area (marine and mangrove ecosystems), and anthropogenic perturbations (road, agriculture, urbanization, and livestock). Environmental conditions and landscape constitution in each area are summarized in Table 1.

Table 1. Ecological, climatic, land use characteristics and genetic information (based on Inter Simple Sequence Repeats (ISSR) markers) obtained at each area for *Eunica tatila* in the Yucatan Peninsula, Mexico. Tree density (d_{trees}), shrub density (d_{shrubs}), density of herbaceous plants (d_{herbs}), sample size (N_1), number of individuals used for analysis (N_2), total number of bands scored over all loci (TB), number of rare bands or bands with frequency ≤5% (RB), number of private bands or bands that exist only in this population (PB), percentage of polymorphism loci (*PPL*), expected heterozygosity (H_e), standard error (SE), and land cover data (forest/coast/anthropogenic) represent the percentage of occupancy of each kind of land cover considered in this study within a 16 km² square surrounding each sampling point as shown in Figure 1.

		Calakmul (CK)	Kohunlich (KH)	Miguel Hidalgo (MH)	Jardín Botánico (JB)	Xcalak (XC)	Kiuic (KU)	Sian Ka'an (SK)	Zapotal (ZP)
Sampling Data	N_1	68	38	17	36	32	59	50	50
	N_2	58	38	17	36	31	54	40	49
Genetic Data	TB	106	98	94	94	95	108	112	104
	RB	17	8	0	9	11	15	5	22
	PB	0	1	0	0	0	2	2	0
	PPL	89	81	76	77	77	90	92	85
	H_e (±SE)	0.194 (0.015)	0.195 (0.016)	0.202 (0.016)	0.187 (0.016)	0.189 (0.016)	0.205 (0.016)	0.220 (0.014)	0.182 (0.016)
Ecological Data	d_{trees}	0.05	0.03	0.05	0.07	0.04	0.08	0.07	0.06
	d_{shrubs}	0.81	1.11	1.37	0.87	1.23	0.89	0.83	1.11
	d_{herbs}	11.38	21.25	12.77	22.33	18.85	40.63	40.12	20.17
	Patch size (km²)	7231	0.085	23	0.65	45	16.5	3750	24
Climatic Data	Temperature (°C)	25.14	27.01	27.01	26.79	27.67	25.51	26.77	26.48
	Humidity (%)	81.04	81.53	81.53	73.42	78.82	79.14	78.94	75.75
	Precipitation (mm)	3.66	5.79	5.79	6.77	6.13	3.19	7.04	1.17
Land cover Data	Forest	98%	93%	79%	24%	13%	100%	100%	98%
	Coast	0%	0%	0%	6%	87%	0%	0%	0%
	Anthropogenic	2%	7%	21%	70%	0%	0%	0%	2%

We sampled a minimum of 30 butterfly individuals per site during the rainy season (from June to August), which corresponds to peak abundance for *E. tatila* [39]. We used 20 Van Someren-Rydon traps [40] per site, baited with banana, pineapple, beer, and sugar [41]. The traps were placed at a height of approximately 2 m during the adult flight period (from 9:00 a.m. to 5:00 p.m.). Most of the attracted nymphalids flying within a radius of 4–5 m around the bait are trapped [40,42]. All *E. tatila* individuals were sexed, kept separately in labeled glassine bags, and geo-referenced (Garmin eTrex 20, Kansas City, MO, USA). Other species of butterflies captured in the traps were released. In the laboratory, the abdomen was cut from each individual, placed in 96° ethanol, and conserved at 4 °C until DNA extraction. Voucher specimens were deposited at the Lepidopterology collection of the "Museo de Zoologia" El Colegio de la Frontera Sur-Chetumal, Mexico.

2.2. DNA Extraction

A small part of the anterior region of the abdomen was cut off, and genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions and stored at −20 °C until amplification. To determine the concentration and quality of DNA, we used a Qubit®2.0 fluorometer (Invitrogen, Carlsbad, CA, USA), and agarose gel (1% with TAE buffer 1X; Promega, Madison, USA) followed by a post-gel staining method using GelRed™ (Biotum, Fremont, CA, USA).

2.3. ISSR-PCR Amplification

The Inter Simple Sequence Repeats (ISSR)-PCR method is employed on plants and animals. This technique is largely employed for genetic population and phylogenetic studies between closely-related species [16,43–46]. It provides highly reproducible results and generates abundant polymorphism in many systems. The advantage of ISSR is that primers work universally for most animal and plant species. Thus, there is no need to define PCR primers for individual species as in microsatellite analysis [47]. Furthermore, this method demands fewer experimental steps and is relatively easy to perform. The use of dominant markers can be considered worthwhile in organisms for which no genomic information are available [48] as is the case for many butterfly species [49]. In addition, microsatellites are inherently difficult to use in butterflies, partially due to the high frequency of null alleles [49]. Therefore, ISSR genetic markers have been widely applied in studies on butterfly population genetic [15–17,43,48,50–57].

We tested 23 different ISSR primers, and selected a total of four that presented good resolution, high number of bands, and permitted a sufficient total number of bands to carry out an analysis [58,59]: $(GAG)_5GC$, $(AG)_8YC$, $(GA)_8C$, $(AC)_8C$ (Table 2). PCR amplifications were optimized as following: 15 µL reaction volume containing ~20 ng of template DNA, 1.5 µL of 5X Green Buffer (Promega), 200 µM of each dNTP (dNTP mix; Promega, Madison, USA), 3 mM $MgCl_2$ (Promega, Madison, USA), 1 µM of primer (Integrated DNA Technologies), 1.25 U of GoTaq Flexi DNA Polymerase (Promega, Madison, USA), and, finally, the volume was adjusted with ultrapure water. All amplifications were conducted in a T100 Thermal Cycler (Bio-Rad™, Hercules, USA) under the following conditions: Initial denaturation step at 94 °C for 4 min, 39 cycles of denaturation at 94 °C for 45 s, annealing temperature (T*a*) from 54 °C to 63 °C depending on the ISSR primer (Table 2), extension temperature at 72 °C for 2 min, and a final extension at 72 °C for 10 min. Amplification products were separated by electrophoresis (110 V for 2 h) using 3 µL of amplified product on agarose gels (2% in a 1X TAE Buffer, Promega, Madison, USA), and a 100 bp DNA Ladder (Promega, Madison, USA) was used as reference for fragment length. The bands were detected with a mix of GelRed™ (20 µL) (Biotum, Fremont, USA) and Blue/Orange Loading Dye Promega (500 µL) under UV light and digitized using an Imaging System (PhotoDoc-it UVP®, Upland, CA, USA).

Table 2. ISSR primers screened for ISSR-PCR in *Eunica tatila*. Percentage of guanine and cytosine content (%GC), melting temperature (T*m*), annealing temperature (T*a*), total number of bands per primers over all localities (*N* bands), size of the DNA fragments for each primer (size). The following designation was used for degenerated sites: Y (C or T).

Abbreviation	% GC	T*m*	T*a*	N Bands	Size (pb)
(AG)$_8$YC	52.8	59.5	58	37	200–1600
(GAG)$_5$GC	70.6	64.8	63	36	400–1400
(GA)$_8$C	52.9	56	54	20	150–1200
(AC)$_8$C	52.9	62.3	61	26	300–1500

2.4. Molecular Analysis

The ISSR markers are treated as dominant markers, amplified fragments were scored as binary information where the presence of a band represents a dominant allele (1), and absence represents a recessive allele (0). Applying this principle, a binary data matrix was generated for all individuals and the four ISSR markers used. Only bands consistent among all individuals were used for analysis.

2.5. Genetic Diversity

We determined the following informative parameters at each locality: Number of private bands (PB) and number of rare bands (RB). The binary matrix was used to assess the standard genetic diversity parameters at each locality and for the whole dataset (Yucatan Peninsula scale): Percentage of polymorphic loci (*PPL*) and expected heterozygosity (*H$_e$*) using corrected allele frequencies [60] implemented in GENALEX 6.5 [61,62]. The differences in *H$_e$* among all localities were evaluated by applying a one-way analysis of variance (ANOVA) using STATISTICA 7.0.

2.6. Relation among Genetic Diversity, Environmental Conditions, and Land Cover

To put in evidence, the relationship among genetic diversity parameters (*PPL* and *H$_e$*) and all environmental (climatic and ecological parameters) and land cover conditions, a multidimensional scaling analysis (MDS) was applied. Furthermore, we quantified the correlations between butterfly genetic diversity (*PPL* and *H$_e$*) and environmental conditions, land cover, and forest patch size with Spearman statistics (*r* and *p*) using STATISTICA 7.0. Finally, to examine the global influence of land cover conditions on genetic diversity (*PPL* and *H$_e$*), we determined the proportional mean values of both genetic parameters for each kind of land cover. Significance among the three levels of land cover was compared with a Kruskall-Wallis test, and, if required, a post-hoc comparison was implemented using STATISTICA 7.0.

2.7. Population Differentiation and Genetic Structure

To evaluate genetic differentiation in the Yucatan Peninsula, and for all population pairs, we determined coefficients of gene differentiation (*G$_{st}$*) and the *PhiPT* (*Φ$_{PT}$*, 999 permutations) using POPGENE 1.32 [63] and GENALEX 6.5 respectively. The *Φ$_{PT}$* parameter (analogous to *F$_{ST}$* and *R$_{ST}$*) is a measure of genetic structuring which allows a comparison between codominant and haploid/binary data [61,62]. Genetic structure and variability within and between populations were determined using a non-parametric analysis of molecular variance (AMOVA) [64] (999 permutations). Isolation-by-distance (IBD) was analyzed by plotting geographic distances (km) against *PhiPT* (*Φ$_{PT}$*) parameters determined between population pairs. The significance of such correlation was tested using the Mantel's test (999 permutations). These analyses were performed using GENALEX 6.5. Finally, to evaluate the genetic structure of *E. tatila*, we applied the Bayesian genetics model implemented in STRUCTURE 2.3.1 [65,66] to infer the potential number of populations in our study. This method is designed to identify *K* clusters for butterfly populations and to assign each individual to one cluster or more, if genotypes indicate intermixing [65]. To determine the number of *K* populations (clusters),

the program was run five times for different values of *K* (from 1 to 8) to allow for deviations among different runs. The admixture and allele correlated frequencies model were used, and we ran the Markov Chain Monte Carlo (MCMC) algorithm with a burn-in period of 10,000 steps followed by another 10,000 steps. To determine the most probable number of *K* that best fits our data, we used a method that consists in identifying the maximum value of Ln*p(D)*, or more specifically the value where the "more or less plateaus" begin [67].

2.8. Sex-Bias Dispersal Analysis

Analysis of IBD by sex was investigated by plotting the geographic distances (km) against genetic differentiation (Φ_{PT}, 999 permutations), followed by a Mantel test to test the significance level of the relationship between both data matrices (genetic and geographic). Analyses were performed using GENALEX 6.5.

3. Results

Out of 350 individuals collected, 323 individuals (between 17 and 58 individuals for each locality, Table 1), were successfully amplified with ISSR primers. The four selected primers produced clear and reproducible fragments with a total of 119 ISSR fragments (or loci) scored (Table 2).

3.1. Genetic Diversity

Considering the whole dataset, the *PPL* was 83.7% (SE = 2.2%) and the expected heterozygosity (H_e) was 0.197 (SE = 0.005). At the locality level (Table 1), the values of *PPL* range from 76% (Miguel Hidalgo locality) to 92% (Sian Ka'an locality), while those of H_e from 0.182 (Zapotal locality) to 0.220 (Sian Ka'an locality). The ANOVA analysis did not present significant differences for expected heterozygosity among all localities ($F_{7,944} = 0.59$, $p = 0.76$). The number of private bands is very low (from 0 to 2; Table 1), confirming that all butterfly samples belong to the same species. The number of rare bands ranges from zero for the Miguel Hidalgo locality to 22 for the Zapotal locality (Table 1). A clear correlation between the number of individuals genotyped and the number of rare bands is observed (Pearson statistics: $r^2 = 0.67$ and $p = 0.013$).

3.2. Relation among Genetic Diversity, Environmental Conditions, and Land Cover

The first dimension of the MDS analysis (Figure 2) shows clearly the positive relation between genetic diversity parameters (*PPL* and H_e) and proportion of undistributed forest, and to a lesser extent, the area size and the density of herbs and trees. Other two land cover conditions (anthropogenic and coastal area), as well as the other environmental conditions (temperature, rainfall, and density of shrubs), show a negative impact on genetic parameters. Spearman correlations indicate that *PPL* has a significant negative correlation only with shrub density (Spearman statistics: $r = -0.71$; $p = 0.048$) and temperature (Spearman statistics: $r = -0.72$; $p = 0.043$), and a significant positive correlation with proportion of undistributed forest (Spearman statistics: $r = 0.92$, $p = 0.001$) (Figure 3). Furthermore, we observed that below 80% of total land cover occupied by forest, values of *PPL* remain similar (approximately 77%), but when the proportion of forest habitat increased from 80% to 100%, an exponential increase of *PPL* values was observed. Additionally, the comparison of the mean values of genetic diversity (H_e and *PPL*) with the proportion of each type of land cover, for all localities, shows that genetic diversity is significantly higher when local populations are surrounded by undisturbed forest rather than in the presence of anthropogenic disturbances or coastal ecosystems (Kruskall-Wallis test for H_e: $H = 13.84$, df = 2, $p = 0.001$; for *PPL*: $H = 13.84$, df = 2, $p = 0.001$) (Figure 4). Patch size does not show a relationship with genetic diversity parameters (H_e and *PPL*; Figure S2).
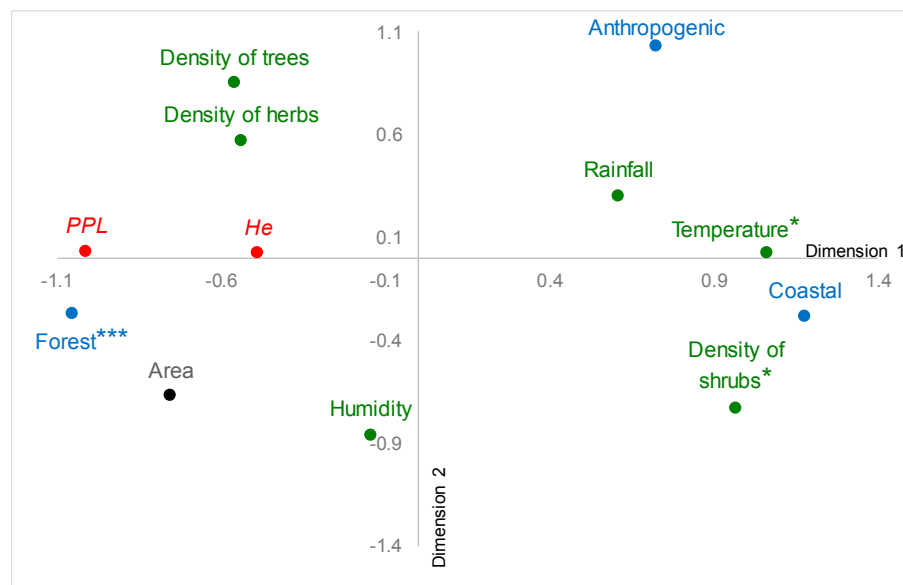
Figure 2. Multidimensional scaling analysis (MDS) considering the genetic diversity parameters in red color (*PPL* and H_e), the land cover conditions in blue (forest, anthropogenic, and coastal areas), patches size in black, and environmental condition in green (humidity, temperature, rainfall, and density of shrubs, herbs, and trees). Significant results of Spearman correlation: *** $p < 0.001$, * $p < 0.05$.
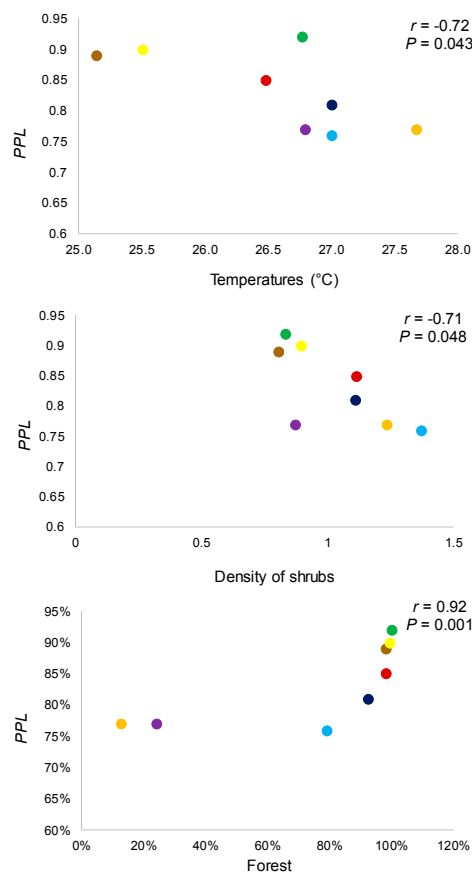

Figure 3. Significative correlation between genetic diversity (*PPL*) and land cover characteristics (temperature, density of shrubs, and forest). Colors represent localities: brown (Calakmul), dark blue (Kohunlich), light blue (Miguel Hidalgo), purple (Jardín Botanico), orange (Xcalak), yellow (Kiuic), green (Sian Ka'an), burgundy (Zapotal). Results of Spearman correlation: *r* and *p*.
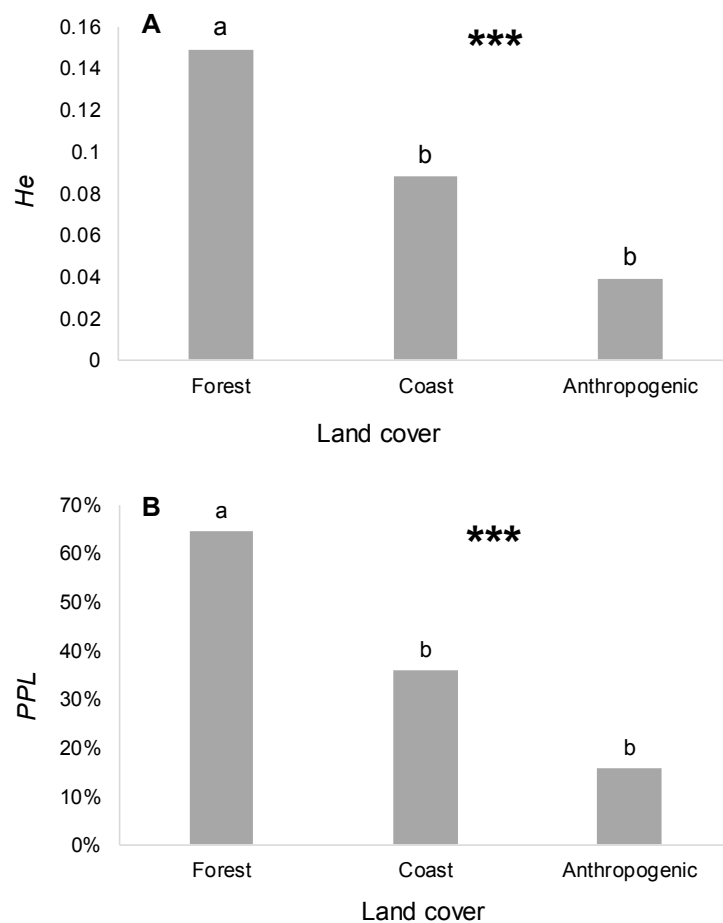
Figure 4. Relationship between the proportional mean value of *He* (**A**) and *PPL* (**B**) and land cover characteristics (forest, coastal, and anthropogenic). Results of Kruskall-Wallis test: *** *p* < 0.05, letters represent results of multiple comparisons test.

3.3. Population Differentiation and Genetic Structure

Genetic differentiation among all localities was very low with G_{ST} = 0.044. Values of Φ_{PT} among pairwise localities were always very low, ranging from 0.095 between the Miguel Hidalgo and Calakmul localities to 0.018 between the Kiuic and Sian Ka'an localities (Table 3). Generally, the Miguel Hidalgo locality presents the highest values of Φ_{PT} with other populations. An AMOVA analysis showed that the principal source of variability is found between individuals within populations (96%), while only 4% of the genetic variability is attributable to diversity between populations (*p* = 0.001). When taking all localities into consideration, an analysis of IBD showed a positive and significant correlation (Mantel test: *r* = 0.039, *p* = 0.006) between pairwise *PhiPT* (Φ_{PT}) values and geographical distances.

Table 3. Coefficient of genetic differentiation *PhiPT* (Φ_{PT}) above diagonal and geographic distances (km) below diagonal for all pairwise comparison. All values of Φ_{PT} are significant at a 0.001 level. Upper and lower values are in bold. For abbreviations of localities see Table 1.

	CK	**KH**	**MH**	**JB**	**XC**	**KU**	**SK**	**ZP**
CK		0.057	0.095	0.042	0.038	0.040	0.042	0.067
KH	115		0.057	0.036	0.021	0.043	0.042	0.050
MH	162	69		0.053	0.056	0.073	0.051	0.085
JB	325	332	264		0.026	0.044	0.053	0.067
XC	217	102	93	306		0.034	0.023	0.030
KU	176	200	178	290	271		0.018	0.030
SK	242	196	126	136	176	212		0.032
ZP	390	342	285	100	355	244	175	

Population structure analysis, using probability assignments estimated by STRUCTURE, did not identify any evidence of structure. Indeed, when we plotted q_i (estimated membership probability) for all individuals when *K* = 2 (Figure S3), all individuals present approximately equal proportions of membership to each cluster.

3.4. Sex-Bias Dispersal Analysis

Only the IBD analysis of females shows a significant correlation (females: Mantel test: *r* = 0.058, *p* = 0.008, Figure 5A; males: Mantel test: *r* = 0.028, *p* = 0.092, Figure 5B).
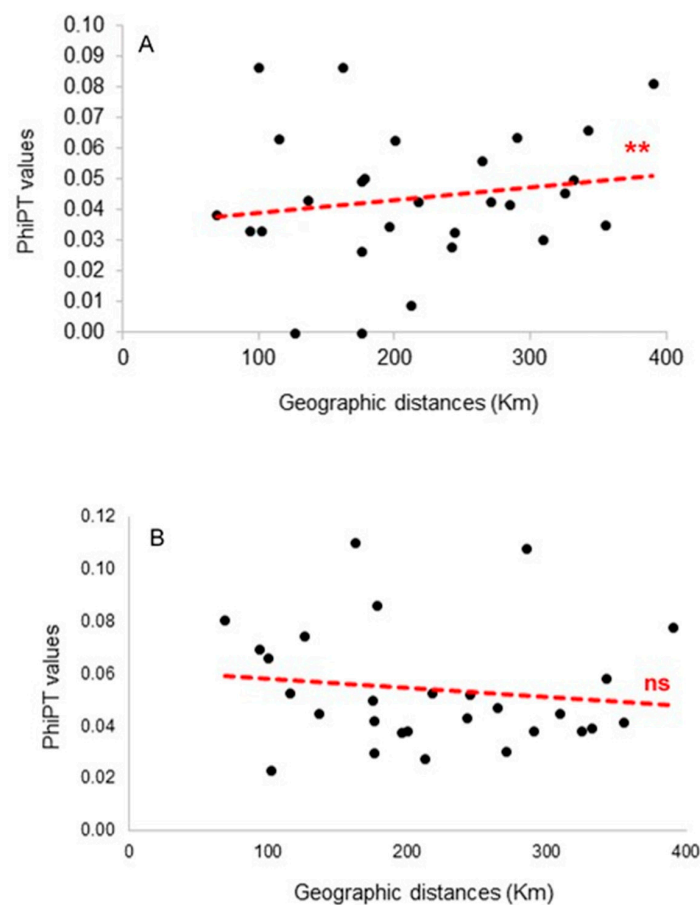
Figure 5. Isolation-by-distance analysis (IBD) for females (**A**) and males (**B**) of *Eunica tatila*. Results of Mantel test: ** *p* < 0.01, ns: not significant.

4. Discussion

Our results reveal that at a wide geographical scale, the *E. tatila* butterfly does not present genetic differentiation among localities, suggesting a metapopulation structure in the Yucatan Peninsula, Mexico. Therefore, although *E. tatila* is a bio-indicator species of undisturbed areas [34], the fragmentation and anthropogenic elements (e.g., cities, roads, crops) do not limit its dispersal among patches of undisturbed forest. Leidner and Haddad (2010) [17] also show that urban zones are not an obstacle to the dispersal of the endemic crystal skipper butterfly (*A. quinteri*). They argue that urbanization does not present a high degree of inhospitableness for this species of butterfly, as it can provide some food sources for adults, in addition to protection from predators and bad weather. *Eunica tatila* is a frugivorous species during its adult stage that feeds mainly on *Manilkara zapota* (L.) P. Royen (Sapotaceae), whereas the host plant reported in Florida for the immature stage is *Gymnanthes lucida* Swartz (Euphorbiaceae) [39,68]. Both plant species are abundant and widely distributed in the Yucatan Peninsula [69], even in cultivated or urbanized areas and along roads, because they are used in different ways by the locals. Ribeiro et al. (2012) [70] observed that the abundance of fruit-feeding butterflies is affected mainly by the immediate fragment surroundings instead the regional landscape as a whole. Furthermore, they emphasized that for the frugivorous butterflies, the action of the people living around the forest patches occupied by butterflies may contributed to its conservation more than the management strategies for the conservation of the population inside the fragment or in a bigger scale. The high abundance of host plants in the Yucatan Peninsula and the observation of Ribeiro et al. (2012) [70] could explain the facility for *E. tatila* to move between patches.

ISSR markers are very useful when studying contemporary changes because they can provide information on genetic structure during very recent isolation processes [45,50,71–73]. The high mobility of *E. tatila*, which is assumed to be partially a migratory species [74–76], together with the lack of geographical barriers in the Yucatan Peninsula and the distribution of the host plants, have resulted in the homogenization of genetic diversity [77], and consequently limiting the process of genetic erosion. Similar results were also demonstrated for other insect species, including butterflies, which demonstrate a high potential for dispersal [17,78,79]. Considering that *E. tatila* presents a metapopulation dynamic inside the Yucatan Peninsula region, we suggest the patchy populations model as defined by Harrison (1991) [80] where the high inter-patch dispersal produce an integrated demographic unit so the local patch population may not be shown as an independent population.

It is generally assumed that specialist species suffer more from the effects of fragmentation than generalist species [81]. Furthermore, the connectivity among patches is more important than the patches size [82], and the population reduction resulting from the habitat fragmentation in specialist species is higher for sedentary species [83]. *Eunica tatila* as a specialized species [84] with a high dispersal capacity, confirms that the degree of specialization is not a key factor in determining the genetic population structure of a species as also shown on bee species in a recently fragmented habitat [79]. Furthermore, Exeler et al. (2008) [85] argue that habitat availability is more important than degree of specialization. The widespread presence of the *M. zapota* and *G. lucida* host plant in Yucatan Peninsula could have provided adequate habitats to favor the dispersal of *E. tatila* in the studied area; therefore, reducing the effects of fragmentation.

The first global IBD analysis (males and females) shows a positive and significant relationship between genetic differentiation and geographical distances. However, the sex analysis shows that males present high dispersal (no IBD) while females show a reduced dispersal capacity (positive and highly significant IBD). Causes of sex-biased dispersal in butterflies have been the subject of previous studies [13]. Trochet et al. (2013) [86] suggested that the principal mechanisms for butterfly dispersal are female harassment by males and male-male competition, rather than the search for a mating partner. Their experimental study also showed that in a male-biased sex ratio population, large-winged individuals present high mortality in the transitional zone (corridor between two potential patches) than short-winged individuals and that females are less successful in reaching

a new patch and surviving after settlement. Our data on *E. tatila* in the Yucatan Peninsula support these hypotheses: All localities sampled present a male-biased sex ratio (Table 1 in reference [37]), and females have wing measurements significantly larger than males (Table 1 in reference [37]). Furthermore, Cavanzón-Medrano et al. (2016) [37] showed that males' thoracic measurements (length and mass) are significantly higher than females, probably an adaptive phenotype of males in a fragmented landscape as suggested by Ducatez et al. (2013) [14] who demonstrated that male flight endurance increases with decreasing habitat connectivity. Therefore, females of *E. tatila* probably present a lower dispersal capacity, due to a high mortality rate when moving between patches and during settlement, influenced by a combination of complex causes such as the population sex ratio, phenotype of individuals, and behavior strategy [86].

The relationship between land cover, environmental conditions, and the genetic diversity of butterflies, has been poorly studied. The majority of studies focus on the effect of global fragmentation on population genetic structure [19,87,88]. It is generally assumed that large habitat patches have higher butterfly populations than smaller ones [89]. Therefore, genetic diversity is expected to be lower in small patches than in large ones, considering that small populations are more susceptible to genetic drift, inbreeding, and deleterious alleles effects [2]. Nevertheless, our results show that patch size, from small (~8 ha) to very large (~700,000 ha), has no effect on genetic diversity. This has been demonstrated for other tropical butterflies such as *Mycalesis orseis* Hewitson, 1864 (Lepidoptera: Nymphalidae) where there was no evidence of a relationship between genetic diversity and forest fragment size or estimated butterfly population size [88]. This was also the conclusion of studies on the Palearctic butterfly, *Polyommatus coridon* Poda, 1761 (Lepidoptera: Lycaenidae) [23,90].

Environmental conditions present a different relationship with genetic diversity. Shrub density and temperature present a significant negative relationship with genetic diversity, whereas the density of tree and herbaceous species, humidity, and rainfall show no correlation with genetic diversity parameters. It is not surprising that temperature shows a significant effect, considering that it has an important influence on all ecological aspects of butterflies, such as distribution, abundance, habitat use, and oviposition site selection among others ([22] for a review). Maya-Martínez et al. (2009) [21] studied the relationship between Charaxinae butterfly population density and climatic and ecological conditions. These authors showed that the key factors for the presence and abundance of different butterfly genera are high humidity and low temperature, as suggested by our results. Therefore, even if variation among temperature seems low (2.7 °C) inside the studied area, this is sufficient to affect the abundance of individuals and consequently, the genetic diversity. Shrub density could affect populations of *E. tatila* considering that a high density of shrubs indicates a lower level of forest conservation and *E. tatila* is a bio-indicator of undisturbed areas. Conversely, high tree density can benefit adults of *E. tatila* as they provide a valuable food resource and individuals settle on tree trunks [39,91].

Our results clearly show the positive effect of undisturbed areas on the genetic diversity of *E. tatila*, confirming this species as a bio-indicator of undisturbed tropical forest. Interestingly, the same positive effect of undisturbed areas has been demonstrated on the diversity of Charaxinae butterflies in the Yucatan Peninsula [21]. An interesting framework (Figure 4) of polymorphism in relation to the percentage of forest cover, showed an exponential increase in values when forest cover exceeded 80%. This suggests that to reach high values of polymorphism, a minimum proportion of forest cover is required for a highly mobile butterfly.

Our study could not draw any conclusions on the possible negative effect of anthropogenic disturbances and coastal areas on genetic diversity. This may be due to the low number of sample points representing diverse types of land cover across the large area of our study. If the number of representative sample points close to the coast and areas subject to human perturbation were increased, we could determine the negative effects of this kind of perturbations on genetic diversity, as suggested by the study of Maya-Martínez et al. (2009) [21] that showed the impact of proximity to coastal

areas (vulnerability due to the impact of hurricanes) and urban settlements, on the distribution and abundance of Charaxinae butterfly species in the Yucatan Peninsula.

Finally, our study shows that a fine-scale analysis of climatic and ecological conditions is necessary to explain genetic diversity variations among a complex tropical ecosystem. Genetic population structure does not appear to be affected by anthropogenic fragmentation, while the availability of host plants inside the matrix is widely distributed facilitating the connectivity between patches for our model species. Nonetheless, we are convinced that increasing studies about this kind of species could lead to stronger results. An increase in molecular studies on tropical butterfly species in fragmented landscapes would lead to a better understanding of the impact of fragmentation on butterfly genetic diversity, and, thus, conservation management strategies.

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