SIGNAL OF CANDIDATE LOCI INVOLVED IN WING COLOR PATTERN IN A *Heliconius cydno* HYBRID ZONE IN CAUCA VALLEY (COLOMBIA)

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ABSTRACT

Heliconius butterflies exhibit a high level of radiation with numerous species that vary in its wing color pattern forms. The importance of these patterns in ecological speciation has been recently documented among these mimetic butterflies. However, the genes responsible for such variation are just starting to be identified in some races and specific populations from Costa Rica and Ecuador, but not in Colombia. Here, a wide genome scan was performed using AFLP (Amplified Fragment Length Polymorphisms), as a new way to approach and detect the signal of candidate genes involved in the production of adaptive traits such as color pattern loci, in natural populations of an *H. cydno* hybrid zone in Cauca valley (Colombia). By performing an AFLP genome scan, a total of 562 polymorphic sites were found using eight different primer combinations, among which only two where related to color pattern elements, loci *Sb* and *Yb*, and could be considered as possible candidates linked to these genes. These results show the utility of AFLPs in finding divergence regions that control characters that may be important in ecological speciation. This and future findings could be used to corroborate if the regions associated with the genes that determine color pattern in Colombian populations are the same as in Costa Rican and Ecuadorian populations.

Key words: AFLPs, ecological speciation, color pattern, *Heliconius cydno*

INTRODUCTION

Ecologically based divergent selection among natural populations can lead to ecological speciation, based on key phenotypic traits involved in resource acquisition. These traits can arise from processes such as the pursuit to obtain food, attract pollinators or predator avoidance (Rundle & Nosil, 2005). Some examples include host choice in the *Timema* genus of walking sticks (Nosil *et al.*, 2008), among others. Previous work on the subject has shown

how ecological divergence, involving key phenotypic adaptive traits, can affect reproductive isolation in these radiations (Feder *et al.*, 1994; Jiggins *et al.*, 2001; Nosil *et al.*, 2008).

Neotropical butterflies from the genus *Heliconius* are a good example of vast adaptive radiation going hand in hand with the color pattern, providing an example of specific key traits involved in ecological isolation (Jiggins, 2008; Chamberlain *et al.*, 2009). This genus, which includes about 46 species (Emsley, 1965; Turner, 1981), has a diverse aposematic color pattern phenotype. It is distributed among different races, where some species have over 30 different subspecies with characteristic Müllerian mimicry rings (Papageorgis, 1975; Brown, 1981; Mallet & Gilbert, 1995). Bright color patterns also function as a warning signal to predators advertising butterfly toxicity and bad flavor. In some cases, these specific color patterns are important in matte choice and hence also responsible for speciation by mimetic shift (Emsley, 1965; Naisbit *et al.*, 2003; Kronforst *et al.*, 2006; Jiggins *et al.*, 2001).

Actually, this genu*s* has been widely investigated in research areas such as adaptation, mimicry, natural selection, genetics, ecology and phylogenetic relationships, using various markers including nuclear and mitochondrial DNA (Brown, 1981; Lee *et al.*, 1992; Brower, 1994a; 1994b; 1996; Brower & Egan, 1997; Brower & DeSalle, 1998; Beltrán, *et al.*, 2002). Furthermore, these butterflies form hybrid zones maintained by frequency-dependent selection (Papageorgis, 1975; Brown, 1981; Mallet & Gilbert, 1995) that constitute naturally appearing laboratories for the study of the forces involved in evolution. One of these species, *Heliconius cydno* is common in Andean forests, distributed from southern Mexico to Ecuador. It has well-differentiated races with distinctive aposematic color patterns. In general, the wing color pattern of *H. cydno* consists in a yellow or white banding pattern over a brilliant iridescent black background. Most of them form mimetic rings with *H. sapho* and *H. eleuchia*, and in some cases with *H. erato* and *Elzunia humboldt regalis* (Ithomiinae). However, some races appear to lack comimic species (Brown, 1979). Manifestation of mimetic and nonmimetic races makes of *H. cydno* interesting species from the evolutionary point of view. For these reasons *Heliconius* constitutes a good biological model to test the importance of ecological speciation in nature.

In the Cauca valley, southwest Colombia, two subspecies of *Heliconius cydno* have been described: *H. cydno cydnides* and *H. cydno weymeri*. The latter is polymorphic and has two distinct forms, *H. cydno weymeri* f. *weymeri* and *H. cydno weymeri* f. *gustavi*. *H. c. cydnides* is found at the north of the upper Cauca valley and mimics *H. eleuchia eleuchia*. On the other hand, *H. cydno weymeri* f. *weymeri* and *H. c. weymeri* f. *gustavi* are restricted to the south of the hybrid zone. Each one forms two different mimetic rings with *Elzunia humboldt regalis* and *H. erato chestertonii*, respectively (Linares, 1996; 1997b) (Figure 1).

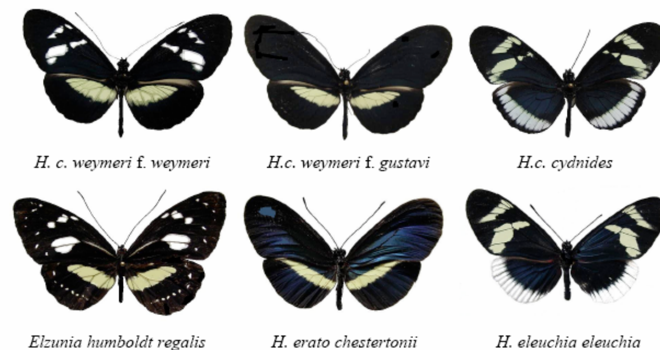

Figure 1. *Heliconius cydno* races from Valle del Cauca and its co-mimics

The color pattern genetic elements of these races were previously described according to cross–fertilization tests (Emsley, 1964; Sheppard *et al.*, 1985; Linares, 1996; 1997a; 1997b; Naisbit *et al.*, 2003). To date, six important loci (L, Sb, Yb, Wo, K and G), that affect coloe pattern in *H. cydno* from Cauca valley, have been reported. On the forewing (FW) the *L* locus controls the total (L_GL_G, L_GL_C) or partial (L_CL_C) presence of melanic scales in the medial area of the FW band. Heterocigous individuals can usually be identified by the presence of white or yellow scales at the FW band between Cu$_1$ and Cu$_2$ veins. Additionally the W_O locus determines the presence (Wo_2Wo_2) or absence (Wo_1Wo_1, Wo_1Wo_2) of a small white oval just below the FW band (Linares, 1996; 1997b). Finally, the locus *K* controls the color of the FW band: dominant allele K^W for white and recessive allele K^Y for yellow. The *L* locus appears to be epistática over *K*: individuals of L_GL_G and L_GL_C genotypeswill always have melanic scales in the FW band independently of the genotype at the *K* locus (Linares, 1996; 1997b; Naisbit *et al.*, 2003).

On the other hand, at the hind-wing, the *Sb* locus controls the presence of the submarginal band (Sb_3Sb_3) located on the hind-wing that is recessive to the absence of this band (Sb_1Sb_1, Sb_1Sb_3). In addition, the *Yb* locus controls the presence/absence of a yellow band (Yb_1Yb_1, Yb_1Yb_2) on the same wing. The two loci *Sb* and *Yb* appear to be closely linked elements with an estimated recombination probability of 1.35% (Merchán, *et al.*, 2005; Joron, *et al.*, 2006a). At last, a minor color pattern element located at the ventral side of the forewing called *G* locus controls the presence (G_2G_2, G_2G_1) or absence (G_1G_1) of a small red line. This last locus does not make any contribution to the mimetic phenotype and is considered probably neutral in this regard (Linares, 1996; 1997a; Naisbit, *et al.*, 2003).

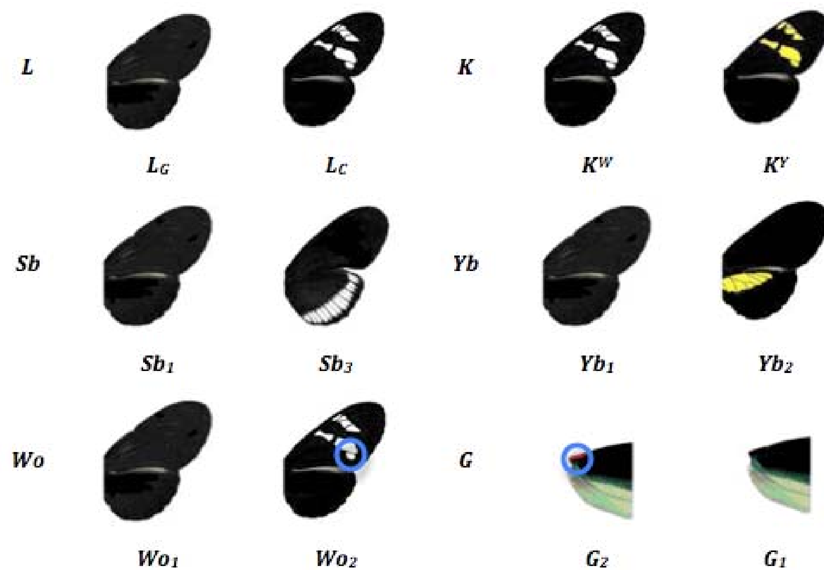

Figure 2. Phenotypic color pattern for each locus. Dominant alleles are located to the left of each particular locus. Top row: Locus *L*, absence of FW band (L_G) and presence of FW band (L_C); and Locus *K*, white FW band (K^W) and yellow FW band (K^Y). Middle row: Locus *Sb*, presence of submarginal band (Sb_3) and absence of submarginal band (Sb_1); and locus *Yb*, absence of yellow band (Yb_1) and presence of yellow band (Yb_3). Bottom row: Locus *Wo*, absence of oval behind FW band (Wo_1) and presence of of oval behind FW (Wo_2); and locus *G*, presence of a red point at the ventral side of the forewing (G_2) and absence of red point (G_1).

The segregation of these color pattern elements (Figure 2) has been determined in previous studies (Linares, 1996; 1997b). However, despite all the research and the great advances in molecular biology, there are many questions to be answered regarding the color pattern. Questions like the genetic basis of wing color pattern and the exact location of the genes

involved in producing particular phenotypes. This information would clarify how the adaptive variation is generated and what loci are involved. One approach to better understand genetics basis of speciation and color pattern in these butterflies is by performing genome wide scans using molecular markers such as AFLP's (dominant markers) (Nosil, *et al.*, 2008). This is a fingerprinting technique that allows investigation of the divergence across the genome by generating many genomic restriction fragments using PCR (Vos, *et al.*, 1995). This investigation attempts to find molecular markers associated with the six genes involved in wing color pattern along a hybrid zone in the Cauca valley, and to detect whether or not is any genotype-phenotype association across the genome of these butterflies.

MATERIALS AND METHODS

Individuals

A total of 169 individuals of *H. cydno* were collected by Mauricio Linares from natural populations at 12 different localities through the hybrid zone in the Cauca valley region (Colombia) (see Figure 3). This analysis included two subspecies of *H. cydno*: and *H. cydno cydnides* in the north, and *H. cydno weymeri* in the south. The latter, as it was mentioned before, is polymorphic and can be found in two different forms, *H. cydno weymeri* f. *weymeri* and *H. cydno weymer*i f. gustavi. Additionally, as a control, an allopatric population of *H. cydno cydno* was sampled in Guaduas, Cundinamarca, for a grand total of 13 localities (Figure 1).

Figure 3. Hybrid zone of *H. c. cydnides* and *H. c. weymeri* in Cauca Valley.

Genotypic characterization

Individuals were visually genotyped for loci L, Sb, Yb, K, G and Wo (Figure 2) according to the observed phenotype, using a stereoscope Stemi DV4 (Zeiss). This procedure was necessary to conduct the search for outliers among the AFLP markers (see below).

AFLP protocols

Genomic DNA was extracted using one third of the thorax tissue for each specimen, following the protocol of DNeasy blood and tissue kit from Qiagen (Valencia, CA). The AFLP data was generated according to Vos et al. (1995) using the AFLP Core Reagent Kit (Invitrogen, modified by Palacio, 2007). Following a preselective amplification, eight selective primer combinations were used to generate fragments: EcoCC-FAM/MseI-CTA, EcoCG-FAM/MseI-CAC, EcoCG-FAM/MseI-CAT, EcoCG-FAM/MseI-CAG, EcoCC-FAM/MseI-CAA, EcoCG-FAM/MseI-CTG, EcoCC-FAM/MseI-CAT and EcoCG-FAM/MseI-CAA (see Table 1 below). The AFLP fragments were sized and scored using ABI GeneMapper software v. 4.0 from Applied Biosystems. Additionally, each locus for all individuals was visually inspected, and those with weak or noisy signal were manually hand called. To guarantee certain degree of repeatability, 18 individuals were genotyped twice for all primer pairs. These replicate samples were run on different plates.

Heterozygosity and polymorphism measures

To observe the level of heterozygosity and variation among the different phenotypic classes, races and hybrids, an analysis using AFLP-SURV version 1.0 (Vekemans, 2002) was performed assuming Hardy–Weinberg equilibrium. Here, the program estimated genetic diversity among phenotypes using a Bayesian method with uniform prior distribution of alleles frequencies (Zhivotovsky, 1999). This method assumes a uniform distribution of alleles frequencies.

Population genetics analysis

Firtly, all polymorphic markers were analyzed with Structure v. 2.3.1 (Pritchard, *et al.*, 2000) assuming a K value of 5 populations, one per each phenotype. It was tested whether or not those clusters identified by Structure corresponded to each phenotypic form. The

analysis was run with a burning in of 10,000 generations and a Markov chain of 100,000 generations with 10 iterations for each K value (2, 3, 4 and 5) using the admixture model under the assumption of Hardy–Weinberg equilibrium. Besides the software recommendations to estimate the value of K, the best K was also calculated using Evanno *et al.* (2005) *ad hoc* statistic ΔK. Furthermore, a principal coordinates analysis (PCA) was performed using the software GENETIX 4.05 in order to reconfirm the results of the other two methodologies (Belkhir, *et al.*, 1996-2004).

"Outlier" loci

To identify outlier loci, the approach used by Beaumont and Balding (2004) was implemented in the program Dfdist. This program generates an empirical distribution of F_{ST} values using a Bayesian method developed by Zhivotvski (1999). Then, a trimmed mean F_{ST} was calculated by removing the highest 30% and lowest 30% of the empirical distribution of F_{ST} values according to Beaumont and Balding (2004) suggestions. Next, a distribution of 50,000 simulated F_{ST} values was generated using a hierarchical Bayesian approach. Finally, the empirical and simulated distributions were compared to identify outlier loci, using the methods described by Beaumont and Balding (2004), and Nosil *et al.* (2008). Here, a number of various comparisons were realized among different groups of individuals. First, to identify outliers concerning the different phenotypic forms a multiple comparison between races was developed, where the hybrids were placed in a single group. Dfdist also generated a F_{ST} value between each phenotypic group (Table 2), which was compared with the structure results. Second, multiple paired comparisons were done between individuals who had the phenotype present and those who did not, for every single locus described above (Figure 2).

RESULTS

Polymorphic markers

A total of 1,111 AFLP markers were obtained ranging between 50 and 400 base pairs. Only 562 markers were polymorphic (50.6%) among the eight primer combinations (Table 1). Those were the only ones used in the remaining analyses.

Table 1. Markers generated by each primer combination

	Primer combinations	Total number of markers	Polymorphic markers
A	EcoCC–FAM/MseI-CAA	112	47
B	EcoCC–FAM/MseI-CTA	97	37
C	EcoCG–FAM/MseI-CAC	140	77
D	EcoCG–FAM/MseI-CAT	171	84
E	EcoCG–FAM/MseI-CAG	155	76
F	EcoCG–FAM/MseI-CTG	141	75
G	EcoCC–FAM/MseI-CAT	138	77
H	EcoCG–FAM/MseI-CAA	157	89

Heterozygosity

The analysis performed with AFLP-SURV v 1.0 (Vekemans, 2002) showed the percentage of polymorphism and the level of heterozygosity for every single race with the hybrids as an individual group (Table 2). Here the proportion of polymorphic loci appears to be relatively high and varies from 64% to almost 81% among the different phenotypic forms. On the other hand, the mean for the level of heterozygosity in different populations is particularly low, with values that not exceed a 30% level.

Table 2. Population statistics computed with the Lynch and Michigan method (1994)

Population	Number of Individuals	Total number of loci analyzed	Proportion of polymorphic loci (%)	Heterozygosity
H. c. cydno	12	556	64	0.24
H. c. cydnides	50	556	77.5	0.23045
Hybrids	44	556	80.6	0.24288
H. c. weymeri f. *gustavi*	42	556	78.6	0.23846
H. c. weyme f. *weymeri*	13	556	80.9	0.26242

Structure analysis

Structure assignment test showed a maximum likelihood value for K = 3 (Ln = -54,655.19). Using the graphical approach described by Evanno *et al.* (2005), the individuals were also assigned to three different clusters as follows: one for *H. c. cydno* individuals, one for *H. c. cydnides*, and one for the two forms of *H. c. weymeri*. Most of the hybrids (22) were closer to *H. c. cydnides*, while some others (13) were closer to *H. c. weymeri* or were a mixture (6 individuals) of both races (Figure 4). These results were consistent with the F_{ST} values obtained through Dfdist analysis (See Table 3 below).

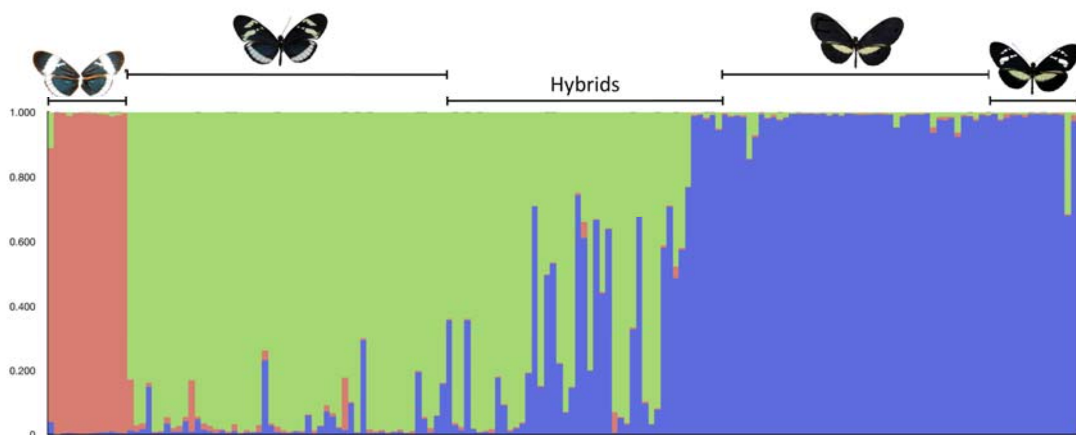

Figure 4. Structure results for 168 individuals from the hybrid zone in Cauca Valley (K = 3).

Principal coordinates analysis

The PCA performed by GENETIX 4.05 (Belkhir, *et al.*, 1996-2004) shows a distribution partially consistent with the previous structure results where *H. c. cydno* individuals were placed in a single group well differentiated from those individuals of the hybrid zone who appear to be grouped in a single cluster.

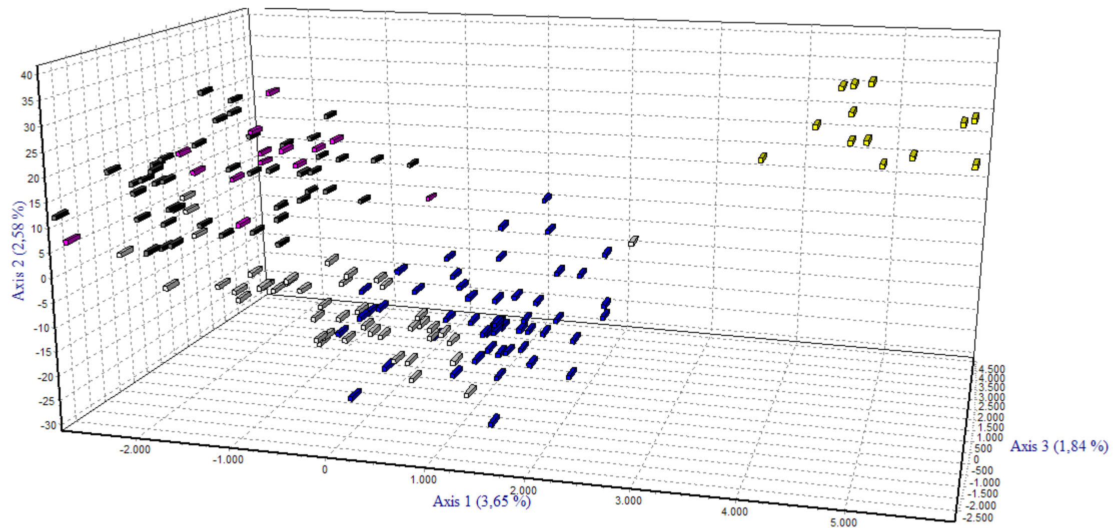

Figure 5. Principal coordinates analysis performed by GENETIX 4.05. Yellow bars represents *H. c. cydno* individuals from Guaduas. The analysis puts *H. c. cydnides* (blue bars), *H. c. weymeri* f. *weymeri* (pink bars), *H. c. weymeri* f. *gustavi* (black bars) and the hybrids (gray bars) as a single group.

"Outliers" loci

A total of 12 outliers were detected in a multiple comparison between the four different phenotypes observed plus the hybrids, using the methods described by Beaumont and Balding (2004) (Figure 6), and Nosil *et al*. (2008) (Figure 7). Both methods show the same results. Additionally, the F_{ST} values for each group where calculated (Table 3). These were consistent with the result obtained in the previous analysis.

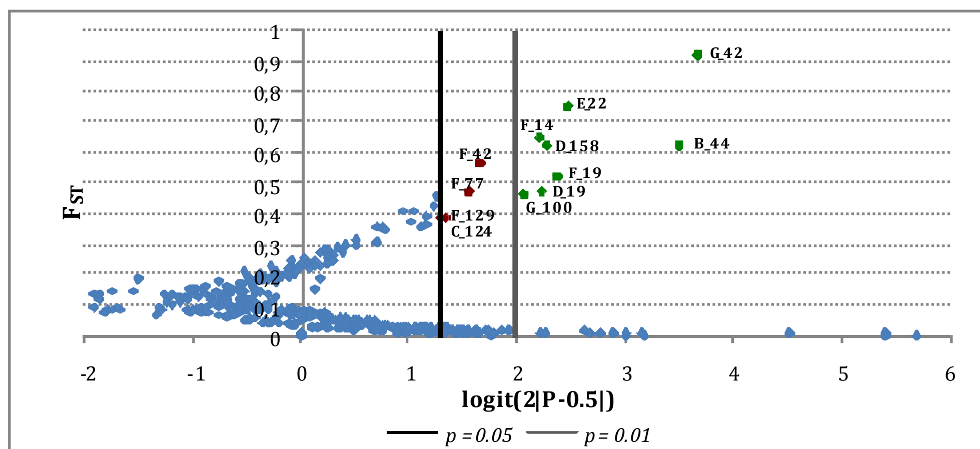

Figure 6. Dfdist analysis results. The plot shows the empirical distribution of F_{ST} values for every single AFLP allele. Vertical bars show the critical *P*–values used to identify outlier loci (Green and red dots).

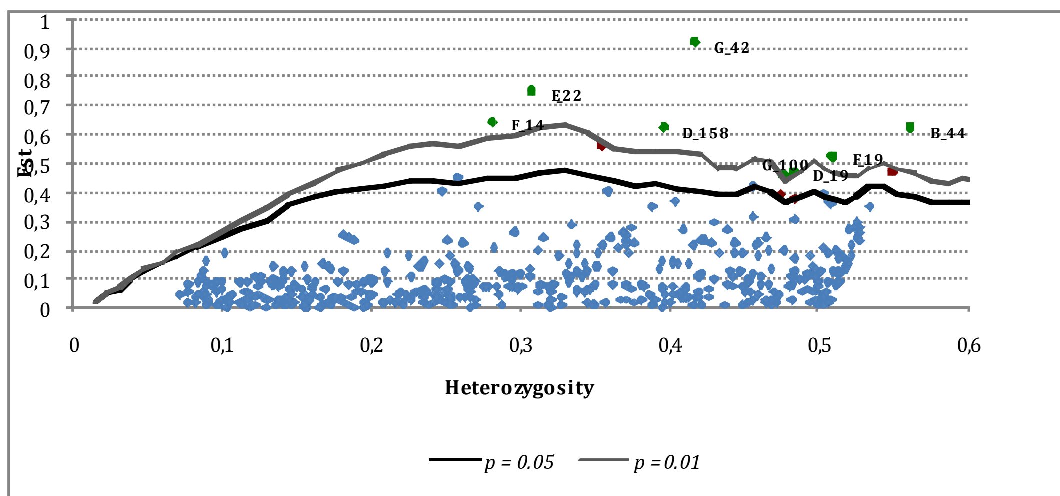

Figure 7. Dfdist analysis results. The plot shows the empirical distribution of F_{ST} values for every single AFLP allele (blue dots) in relation to a 95th quantile (black line) and 99th quantile (gray line). Highly significant outliers (green dots; $p < 0.01$) are labelled. Red dots represent significant outliers ($p < 0.05$)

Table 3. F_{ST} values obtained with Dfdist between each group.

	H. c. cydno	*H. c. cydnides*	*Hybrids*	*H. c. weymeri* f. *gustavi*	*H. c. weymeri* f. *weymery*
H. c. cydno	-	0.153312	0.182350	0.202570	0.202513
H. c. cydnides		-	0.027205	0.063443	0.065806
Hybrids			-	0.035075	0.044002
H. c. weymeri f. *gustavi*				-	0.0
H. c. weymeri f. *weymeri*					-

Finally, when multiple paired comparisons by phenotype were done, only 1 AFLP band, number 44, from primer combination B (EcoCC–FAM/MseI-CTA) continued to appear. Band B_44 was found to be an *"outlier"* which appears to be related with both characteristics, the absence of the submarginal band *Sb* ($p < 0.01$) and the absence of the yellow band *Yb* ($p < 0.05$) over the dorsal surface of the hindwing of these butterflies. On the other hand, one particular AFLP band, number 16 from primer combination F (EcoCG–FAM/MseI-CTG) shows a significant relation ($p < 0.05$) with the presence of the submarginal band *Sb*, but it was not identified as an *"outlier"* when the multiple population comparison was done (Figure 8).

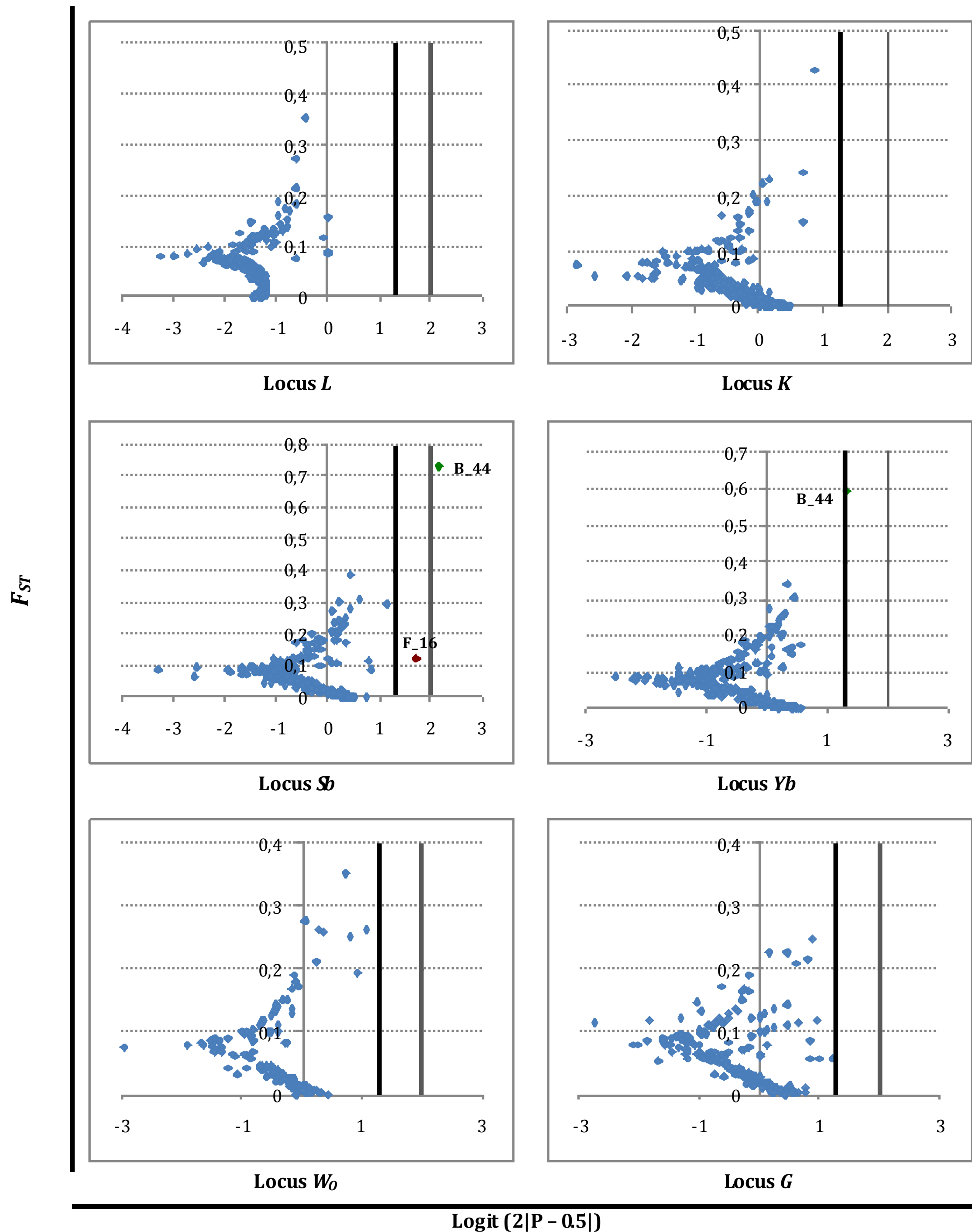

Figure 8. Fdist analysis results. The plots show the empirical distribution of F_{ST} values for every single AFLP allele. Vertical bars show the critical *P*–values used to identify outlier loci (Green and red dots): black bar for a p = 0.01 and gray bar for p = 0.01.

DISCUSSION

Previous studies on *Heliconius cydno* hybrid zone in Cauca valley have shown that most of the phenotypic variation in color pattern is determine by a few loci of great effect (*L*, *K*, *Sb* and *Yb*) and other minor loci (W_O and *G*), all of mendelian inheritance. Past work also described the contact zone as a narrow hybrid zone in which the hybrids are very common, even more frequent than the parental races, with color pattern genes in Hardy–Weinberg equilibrium and almost non-existing linkage disequilibrium, suggesting random mating (Linares, 1996; 1997a; Merchán, *et al.*, 2005).

The population genetic F_{ST} values over this genome scan showed moderate genetic divergences among the *Heliconius* races inside the hybrid zone (larger than 0.05), in comparison with anyone of them and the allopatric population of Guaduas (Table 3). However there was no genetic differentiation between the two distinct forms of *H. c. weymeri* ($F_{ST} = 0$). Moreover, clustering of the AFLP data with the program Structure v.2.3.1 (Pritchard, *et al.*, 2000) failed to detect any color pattern associated in this polymorphic races suggesting high levels of gene flow among them, despite the differences in its coloration. In addition, PCA analysis performed by GENETIX v. 4.05 (Belkhir, *et al.*, 1996-2004) shows a continuum across the hybrid zone with slight separation between the pure races, but not between the two forms of *H. c. weymeri*. PCA shows as well that the variation among them is not really great (Figure 5, Table 2) where the axes explain less than 4% of the observed variation. These also can be seen in the low mean level of heterozygosity for the different populations (less than 30%). Results reflect a low degree of variation, which can be consequence of free gene flow. Only the population of Guaduas was clearly separated from the other races in all the analysis. These could be due to the fact that this is an allopatric population on the other side of the mountain chain, which would make genetic flow difficult between it and the races involved in the hybrid zone.

Therefore, despite the fact that the phenotypic transition across the hybrid zone appears to be very sharp, with distinct forms with particular color pattern, at the AFLP level this distinctiveness is not detected. These could be consequence of current free genetic flow among races that reflects on the high frequency of recombined genotypes (typical

characteristics of a unimodal hybrid zone). However, this result is not surprising because the loci obtained here could be neutral markers not subject to selection pressures, allowing them to pass freely across the hybrid zone. The results obtained at the present work along with previous research confirm that the races involved have not yet diverged enough for the development of prezygotic or postzygotic barriers that effectively avoid the production of hybrids (Jiggins & Mallet, 2000). This is expected considering that these are all races of a same species. Although in recent studies about a hybrid zone in Costa Rica races show strong local genetic differentiation between the two species involve, *H. pachinus* and *H. c. galanthus* (Chamberlain, *et al.*, 2009).

However, here the genome scan was able to detect twelve *outliers* (2.13%) in multiple population comparisons (Figures 6 and 7). These represent a small proportion of the genome most likely subject to divergent selection separating the *H. cydno* races. Furthermore, two *outliers* that could be associated to color-pattern loci (less than 1%) were found: loci B_44 and F_16. These AFLP alleles constitute strong candidates linked to *Sb* and *Yb* genes (Figure 8). The case of locus B_44, who came out as an *outlier* in three different comparisons is particularly curious. It was found to be strongly associated with both dominant traits, the absence of submarginal band *Sb* locus ($p < 0.01$) and the absence of yellow band *Yb* ($p < 0.05$). Nevertheless, earlier investigations report these loci as closely linked elements with an estimated recombination probability of 1.35%, that are almost always found in repulsion association and the combination of both traits at the same time are highly unusual (Merchán, *et al.*, 2005; Joron, *et al.*, 2006a). Hence, it is quite possible that this particular AFLP band is not precisely associated with these two color pattern elements, but to a divergent region inside the genome different between *H. c. cydnides* and *H. c. weymeri* that could arise as a consequence of selection. Actually this marker is present in a high frequency on *H. c. weymeri* (78%) and absent in the former race. On the other side, AFLP allele F_16 shows significant association with the absence of the submarginal band ($p < 0.05$) but no with absence of the yellow band. For this reasons this is a particularly strong and good candidate to be associated with the genome region linked to both loci. Therefore, it could be used to locate and characterized the genes involve in these traits, as was done previously by Baxter *et al.* (In press). In that study, the researchers performed three pair–

wise population comparisons, two involving races of *H. melpomene* (*amaryllis* vs *aglaope* and *melpomene* vs *rosina*) and one pair of hybridizing species (*H. pachinus* vs. *H. cydno*). They identified 30 predicted genes across the 330 kb of *H. melpomene Yb* locus. Among them, the strongest peaks of association were around the *HM00023* and *HM00024* genes, both between the species comparison of *H. cydno* vs. *H. pachinus* and between *H. m. amaryllis* vs. *H. m. aglaope* (Baxter, *et al.*, In press). However, even though great effort and time have been taken to locate this zone, there is still much to be done to find the precise sequence, besides the fact that there are no studies in Colombian populations to corroborate these results.

For this reason, the present work is an alternate approach to the same problem, that could be use to corroborate previous works. Here, by detecting outlier AFLP markers possibly associated with color pattern, we can compare our findings with those of other zones. In this way we can verify if the same genes are involved in both hybrid zones. Nonetheless, the present findings need to be confirmed with the analysis of other populations and more AFLP loci. Hence, by performing a more exhaustive genome scan with more primer combinations we can see with more clearness across the genome. This way we can increase the chance to find out more color-pattern associated *outliers* that help us to locate the genes involved in color pattern (specially those not yet found), which are very important in the process of ecological and adaptive radiation of these butterflies. Besides, in a near future mate preference assays could be performed to elucidate if the color pattern in these races of *H. cydno* is important in premating isolation, like in Costa Rica populations (Chamberlain, *et al.*, 2009). If an association between ecologically based signal like color pattern and mate recognition is found, these could explain the rapid radiation of forms and races in these butterflies.

CONCLUSIONS

This work manages to find two *Outlier* loci involved in the divergence of these butterflies, one of which is strongly associated with a color pattern element (the presence of the submarginal band). Since these traits are evolutionary important not only in *Heliconius cydno*, but among another species, the finding and analysis of regions controlling color

pattern elements will give us some light about the evolutionary processes that lead to the ecological speciation of these butterflies. These results show how the AFLP technique is a good and complementary approach to the search of highly variable regions involved in the divergence and evolution across different lineages. And what is more important, the methodology used at the present work could be extrapolated to other groups of which nothing is known about its genetic architecture and adaptive evolution.

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