Patterns of genetic and morphological diversity in the highly polymorphic Neotropical banner damselflies, *Polythore* (Polythoridae:Odonata).

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Title: Patterns of genetic and morphological diversity in the highly polymorphic Neotropical banner damselflies, *Polythore* (Polythoridae:Odonata).

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ABSTRACT

Genetic divergence across populations can be favored by geographical and/or phenotypic processes. However, phenotype polymorphisms caused by natural and/or sexual selection can obscure patterns of genetic divergence due to disparity across the morphological or behavioral traits with the genetic makeup in the populations. The Neotropical banner damselflies of the genus *Polythore*, exhibit a striking wing color polymorphism across all its geographical range, which includes the Andes Cordillera and Amazon Basin. The latter suggests that they are excellent model organisms to test the effects of high phenotypic polymorphisms and geographical processes on the patterns of genetic diversity. Our aim was to explore the genetic and morphological diversity across the phylogenetic tree of these colorful damselflies. Our mtDNA phylogenetic reconstruction shows a strong association with geographical location, resulting on the recovery of four well-supported geographical clades (i.e: Amazon clade; and the West, Southeast and Northeast Andean clades). Overall, the patterns of genetic and morphological diversity are high and concordant across all members tested in the Amazon clade; suggesting that these population may have been experiencing divergence due to vicariant events. While for the Andean clades, the morphospecies showed a pattern of recent diversification that might be promoted by dispersal events. Finally, the wing color pattern seems to be shaped by other selective pressures including, sexual and/or natural selection.

Key words: Odonata, cryptic species, polytypic species, geometric morphometrics, vicariance, mimicry.

RESUMEN

Procesos de divergencia genética pueden ocurrir debido a procesos geográficos y/o fenotípicos. No obstante, los polimorfismos fenotípicos causados por selección natural y/o sexual, pueden dificultar encontrar patrones de divergencia genética debido a diferencias entre rasgos genéticos y morfológicos. Las libélulas del género *Polythore* han demostrado polimorfismos llamativos de color alar que se encuentran a lo largo de su distribución geográfica, la cual incluye la cordillera de los Andes y la cuenca Amazónica. Lo anterior sugiere, son excelentes modelos poner a prueba los efectos de altos polimorfismos fenotípicos y procesos geográficos sobre los patrones de diversidad genética. Nuestro objetivo fue explorar la diversidad genética y morfológica a lo largo de la filogenia de este colorido género. La hipótesis filogenética obtenida para tres loci de mtDNA muestran una asociación estrecha con la geografía, definiendo así cuatro clados geográficos altamente soportados (i.e. Amazónico, Oeste, Noreste y Sureste de los Andes). En general, los patrones de diversidad genética y morfológica del clado Amazónico, fueron altos y congruentes entre las morfoespecies, sugiriendo patrones de divergencia explicado por posibles procesos. Mientras que las morfoespecies Andinas mostraron un patrón de diversificación reciente el cual puede ser explicado por eventos de dispersión. Finalmente, la diversificación del patrón de coloración alar de *Polythore* parece ser promovida por presiones de selección que pueden ser sexuales o bien, naturales.

Palabras Clave: Odonata, especies crípticas, especies politípicas, morfometría geométrica, vicarianza y mimetismo.

INTRODUCTION

A fundamental goal of phylogenetics and systematics is to recover monophyletic clades, although populations and species are continuously shaped by multiple biotic (e.g. gene and ecological interactions) and abiotic (e.g. climate, topography) variables making this task not a trivial one (Lowry & Gould, 2016). The process of species formation, speciation, is understood as a continuous sequence of genetically based events that happen as two lineages diverge from another on the path to reproductive isolation (Lowry & Gould, 2016; Orr, 1995; Sobel, et al, 2010) . Sometimes this isolation can be product of, vicariance events, which are the appearance of geographical barriers that prevent gene flow between populations over time (Coyne, 1998). However, intrinsic dispersal abilities of the organisms over the landscapes can also promote either, gene flow or reproductive isolation (Zink, et al., 2000) . For example, in the Neotropical region, the Andes Cordillera has played an important in shaping the diversity due to vicariance and dispersal events in multiple organisms including, plants (Antonelli, et al., 2009; Heads, 2019), birds (Chaves, Weir, & Smith, 2011; Winger et al., 2015), frogs (García-R et al., 2012; Guarnizo, et al., 2009), and insects (De-Silva, Elias, Willmott, Mallet, & Day, 2016; Dick, Roubik, Gruber, & Bermingham, 2004). However only few studies on fish have looked at the Andes role in freshwater water ecosystems (Rincon-Sandoval, Betancourt, & Maldonado-Ocampo, 2019), suggesting the need to evaluate diversification process in other freshwater organisms.

Dragonflies and damselflies (Odonates) are one of the most ancestral and conspicuous orders of insects. They are part of both, freshwater (i.e. nymphs) and terrestrial (i.e. adults) ecosystems, making them an unexplored model to look at genetic diversity and isolating barriers. The Neotropical damselflies of the genus *Polythore* have extremely colorful wing color patterns and it belongs to the family Polythoridae (Odonata:Zygoptera). It comprises 58 species distributed on 6 genera: *Chalcopteryx*, *Chalcothore*, *Cora*, *Miocora*, *Euthore* and *Stenocora* (Herrera et al., 2018). Traditionally, its taxonomy has relied in morphological characters such as wing coloration and the secondary male structures (i.e. ligula) (G. H. Bick & Bick, 1985, 1990, 1986, 1992). Although the wing color characters have demonstrated to be uninformative for species delimitation, either because they are highly variable within a group, or invariant among groups (Sanchez et al. 2010, 2015). Suggesting, that the wing color pattern might be due to phenotypic

convergence, raising the possibility that this genus has both, polytypic and cryptic species within the genus.

In this study we explored the effects of high phenotypic polymorphisms (e.g. wing color patter) and geographical processes on the patterns of genetic diversity across the phylogenetic tree of these colorful damselflies. Using phylogenetic, population genetics and morphometric analyses we were able to observe that most of the genetic variation present in this genus in explained by geography. However, depending on the clade, it was either a vicariance or dispersal events, which promotes the different patterns of the genetic and morphological diversity.

MATERIALS AND METHODS

Taxon Sampling

We collected a total of three hundred and forty-five (345) *Polythore* specimens across multiple locations in the Andean foothills and Amazon basin of Colombia, Ecuador and Peru (Fig. 1, for details see Supplementary Table 1). After collection, specimens were kept on individual glassine envelopes that were stored in airtight dry containers or in vials with DMSO 100% or absolute ethanol solution to preserve the tissue.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from thoracic muscle using the DNeasy Blood And Tissue Kit, following the manufacturer protocol, except for the following variations: a prolonged incubation of the samples in Proteinase K at 56 °C for 24h; and we eluted the DNA from the spin column with 100 uL to increase DNA yield. We amplified three mtDNA fragments, two protein coding regions: *Cytochrome Oxidase I* (COI) and *NADH dehydrogenase I* (NDI), and one ribosomal subunit (16S). We used the following primers pairs for each of the genes: C1J - TL2 for COI (Simon et al., 2015), 16sF - 16sR for 16s (Sanchez Herrera et al., 2018) and P850 and P851 for NDI (Hadrys, Balick, & Schierwater, 1992). All PCR protocols are described in the Supplementary Material Appendix 1. We cleaned all the PCR products for sequencing using the NucleoSpin Gel and PCR purification kit (Macherey-Nagel, 2011). Sanger Sequencing reactions were performed bidirectionally at MACROGEN Inc. laboratories (Seoul, Korea). Forward and reverse sequence strands were assembled and edited using GENEIOUS PRIME (Biomatters

Limited, 2018). Finally, we created three multiple sequence alignments for each gene fragment using MUSCLE (Edgar, 2004) plugin in GENEIOUS PRIME and then were manually verified using Mesquite (Mesquite Project Team, 2014).

Phylogenetic Reconstruction

To recover the phylogenetic position of the genus *Polythore* within the family Polythoridae we obtained all the sequences of all the other genera and outgroups in the family previously published by Sanchez et al. 2018 (see Supplementary Table 1 for the accession numbers). We performed a Maximum Likelihood Analysis using the software IQTREE (Nguyen, et al., 2015) . We ran a partitioned analysis by fragment and use IQTREE internal ModelFinder to obtain the best substitution models for each of the partitions using the Bayesian Information Criteria (BCI). We performed a branch support analyses using 1000 pseudoreplicates of Ultrafast Bootstraps (Minh, Nguyen, & Von Haeseler, 2013) and a single branch test of SH-aLRT (Shimodaira & Hasegawa, 2001) . Finally, the best topology was visualized and edited in Fig.Tree (Hancock, Zvelebil, & Cummings, 2014) .

Population genetics analyses

To explore the patterns of genetic variation of all the gene fragments in the genus *Polythore,* we assessed the haplotype diversity and polymorphism statistics (i.e. number of haplotypes (h), genetic diversity ($\theta\pi$), and genetic diversity per segregating sites ($\theta(S)$) of representative species (see Table 1.) within the phylogenetic clades recovered in our ML reconstruction using the population genetics suite Arlequin v3.5.2.2 (Excoffier & Lischer, 2010) . We applied a network representation of the haplotype relationships, including unsampled haplotype variants. Thus we calculated and visualized Minimum Spanning Networks (MSN) in the population genetic software package POPART (Leigh & Bryant, 2015) . Finally, to determine the degree of population structure between all the geographical clades for each species, pairwise F_{ST} values and pairwise genetic distances were calculated in Arlequin and 95% statistical significance for each test was obtained by 1000 randomizations.

Wing Geometric Morphometric Analyses

To explore the variation in wing color pattern across *Polythore*, we performed a landmark analysis of the same species selected on the population analysis. Here we followed the protocol described by (Herrera et al., 2015). For doing so, we scanned a total of 36 fore (hereafter, FW) and hindwings (HW) of the recent collected populations of *P. beata* and *P. procera* using an EPSON Scanner Perfection V550 and the scanning template of the TOWD project (Kunh et al. in prep). All images were scanned at 1200dpi resolution. We placed a total of 50 landmarks, using ImageJ (Rasband, 1997) Point Picker plugin (Supplementary Appendix 2.). All the X,Y coordinates obtained were converted into TPS files and then combined with the TPS files previously published by (Herrera et al., 2015) . Overall, we landmarked a total of 218 forewings (i.e. Amazon 31FW, West Andes 11FW, SE Andes 78FW and NE Andes 98FW) and 128 hindwings (i.e Amazon 31HW and SE Andes 97HW). We only used FW for the West and NE Andes because both wings share a similar pattern. We performed a PCA analyses for FW and HW independently in the R package GeoMorph (Adams & Otárola-Castillo, 2013), the PC loadings were extracted to determine which parts of the shape of color pattern show the highest variation. Finally, we used a MANOVA in R to test if there were significant differences among the sexes, species and/or population for each target group tested.

RESULTS

Phylogenetic reconstruction

Our phylogenetic reconstruction is consistent with previous studies (Sanchez et al. 2018; 2015). We recovered all the genera clades, *Chalcopteryx* (96.2% BS), *Cora* sensu stricto (99.4% BS), *Miocora* (85% BS), *Euthore* (97.2% BS) and *Polythore* (99.8% BS). Particularly, the inclusion of the new *Polythore* populations didn't change significantly the relationships within the genus. We recovered two reciprocal monophyletic clades showing geographical correspondence to the Andes mountain range (99% BS) and the Amazon basin (100% BS) (Fig. 2B). The populations sampled in Leticia (RNNP) and in La Pedrera were nested within the Amazon Basin Clade, however they are not sisters to each other, Leticia was recovered sister to *P. mutata* (Tiputini) and La Pedrera (99.8% BS, Fig. 2B). we present an extended amazon clade which consists of Polythore specimens from the Ecuadorian, Peruvian and Colombian Amazon basin. Within this

Amazon clade (Fig. 2B): Ecuadorian *P. aurora* specimens were clustered in a highly supported monophyletic clade (100% BS), sister to the strongly monophyletic (99% BS) extant specimens *P. beata* specimens from Colombia and Ecuador.

On the other hand, the Andes Clade comprises two monophyletic clades the West Andes (68% BS) and Eastern Andes Clades (99% BS, Fig. 2B). All clades recovered the same species complex previously reported by Sanchez-Herrera et al. 2018. The Eastern Andes Clades shows paraphyly of multiple species within the clade (Fig. 2B). The population of Santa Maria, Boyaca was recovered sister to the Guayabetal population in a cluster of *P. procera* sister to the extant species of the NE clade (98% BS, Fig. 2B).

Population genetics analyses

Genetic diversity patterns

The haplotype networks show variable patterns of genetic diversity across the targeted species in each of the geographical clades in *Polythore* (Fig. 4). However, all the mtDNA fragments were consistent within each clade. For the Amazon clade we selected all members within it, due to low population sampling we have for each morphospecies. For COI, we observed well-structured minimum spanning haplotypes for each population, showing the highest haplotype diversity (see Table 1) for the Tiputini population representing the species *Polythore mutata* (Fig. 4). There are almost 63 mutational steps between Leticia (RNNP) and Iquitos (*Polythore aurora)*, while there are 29 steps between Leticia (RNNP) and La Pedrera both morphologically classified as *Polythore beata.* In contrast, Leticia and Tiputini have only 10 steps in between supporting the phylogenetic hypothesis (Fig. 2.B) which recovered as sister taxa. For the mtDNA ribosomal 16S fragment the haplotype network shows a similar haplotype diversity, although the in this case la La Pedrera population has 19 mutational steps with Leticia and both have 34 mutational steps with Tiputini (see Supplementary Figure. 2). For ND1, we didn't have individuals for *Polythore aurora*, but we recovered a similar pattern than COI (see Supplementary Figure. 2). Our polymorphism statistics for all the population for each gene fragments shows different patterns of genetic diversity, but consistent patterns with the haplotype networks (see Table 1).

In contrast, the Andean clades show dissimilar patterns of genetic variation across all the geographical clades. For the West Andean clade, represented by the morphospecies Polythore gigantea, we explored three Colombian (i.e. La Miel, La Doctora and Copacabana) and one Ecuadorian populations (i.e Mindo). For COI, we observed that "La Doctora" and few individuals of "La Miel" share a one haplotype (Fig. 3.B). We observed well-differentiated haplotypes among the Colombian and Ecuadorian populations with 19 mutational steps of separation. However, the populations with highest haplotype diversity are "Mindo" and "La Doctora". For 16S and ND1, we recovered a lower haplotype diversity in comparison with COI, although we observed two different haplotypes for the Colombian and Ecuadorian populations (Supplementary Figure. 2, Table 1). For the Southeastern clade, represented by two morphospecies *P. neopicta* and *P. victoria*, we analyzed four very close populations along a road of the Central Andes of Peru (i.e. Pozuzo1, Pozuzo2, Pozuzo3 and Santa Cruz). In this clade the haplotype diversity is higher than the observed in the Amazon and West Andes Clades for the COI and ND1 fragments (Fig. 3B, Table 1). However, for both fragments most of the variation is contained within the "Pozuzo 1" population (see Table 1.). For 16S, we observed only two haplotypes separated by 2 mutational steps corresponding to the "Pozuzo" populations and "Santa Cruz". Finally, the high genetic diversity in our sampling was obtained in the NorthEastern clade (Supplementary Figure. 2, Table 1). Represented by five Colombian (i.e. La Catira, Chirajara, Santa Maria, Cementerio, Bavaria) and one Ecuadorian (i.e. Rio Negro) populations of the morphospecies, Polythore procera. For COI, we recovered a total of 23 haplotypes. The "Chirajara" and "Rio Negro" populations are well separated from the other populations by 21 and 48 mutational steps respectively (Fig. 3.B). Although, between them there were only 6 changes. For 16S, "Santa Maria" is well separated from the other populations by more than 30 steps. While for ND1, the haplotype network shows shared haplotypes among the populations. Our genetic diversity estimates show that "Santa Maria" is the population with the highest diversity, although our sample size might be affecting those results (see Table 1).

Population Structure

Our estimates of population structure are in agreement with the patterns of genetic diversity of the target species within the geographical clades. For the Amazon clade, we found high and significant F_{ST} values among most of the populations tested (Fig. 4). Although, the Iquitos and RNNP populations show less structure in the COI gene, and almost zero in the 16S, this could be

an underestimation due to our low sample size. For the Andean clade, the NW showed a high and significant F_{ST} values among all the comparisons between the Ecuadorian and the Colombian populations in all the gene fragments (Fig. 4). However, within the populations in, Colombia and Ecuador, the F_{ST} values were low. For the SE, we recovered a significant genetic structure between "Santa Cruz" and all three populations for "Pozuzo", and a low structure among all Pozuzo populations. At last, the NE clade, *Polythore procera*, a variable pattern of genetic differentiation in the gene fragments (Fig. 4), which are consistent with the paraphyletic relationships obtained in the phylogenetic approach (Fig. 2.B). In addition, the pairwise distances (Pixy) for all the clades showed consistent results (Fig 4.). In general, we found structured populations.

Morphometric Analyses

The PCA analyses of the wing color pattern in both, fore (FW) and hindwings (HW), showed differences in all the target morphospecies tested in each the clade. The Amazon clade shows different wing color patterns for FW and HW (Fig. 3.A & 5). Eighty three percent of the variation of the FW wing shape was explained by the two first PCA's, PC1 (63%) and PC2 (20%) (Fig. 5, Table 2). PC1 resumes the variation in the landmarks of the overall size and the band V (Table 2), while PC2 explains the landmarks of the bands at the tip of the wings. On the other hand, the PCA of the HW explained 81% of the variation in PC1 (53%) and PC2 (26%). Which resume the variation in size and the position of bands I to III in PC1, while for PC2 resumes the variation in the bands IV and V (Table 2.). The MANOVA shows no significant difference on the shape variables in the FW (PC1 and PC2) by sex (PC1, F=0.4286, $p=0.5^{n.s}$; PC2, F=0.0019, $p=0.96^{n.s}$), but significant values by morphospecies (PC1, F=481.55, $p=2.2 \times 10^{-16}.^{**}$; PC2, F=6.54, $p=0.0046^{**}$). The HW MANOVA shows significant differences in the new shape variables for both sex (PC1, F=4.89, $p=0.03^{*}$; PC2, F=10.7, $p=0.002^{**}$) and morphospecies (PC1, F=22.62, $p=1.4 \times 10^{-6}.^{**}$; PC2, F=6.5, $p=0.0047^{**}$, Table 2).

In the SE Andes clade the wing shape of color pattern, *Polythore neopicta* and *P. victoria*, was not able to differentiate among the morphospecies in comparison with the Amazon clade (Fig. 3A & 5). For the FW, the two first PC's were able to resume the 72% of the variation and for the HW only the 56% of the variation. The FW PC1 explains 49% and resumes information of the

wing size and shape of the bands IV and I (Table 2.); while for PC2 explains 23% of the wing size and the band III (Table 2.). For the HW, the PC1 and PC2 explains 37% and 19% respectively and both resumes the shape and size of the band II (Table 2, Figure 3A & 5). The MANOVA shows significant differences in the new shape variables for both sex (PC1, F=13.14, p=0.0005***; PC2, F=120.03, p=2.2 x 10^{-16}.***) and species (PC1, F=6.28, p=0.014**; PC2, F=4.13, p=0.045*) in the FW. The MANOVA for the HW shows no significant differences on the shape variables (PC1 and PC2) by sex (PC1, F=58.38, p=1.7 x 10^{-11}; PC2, F=3.12, p=0.08$^{n.s}$), but significant values by morphospecies (PC1, F=8.58, p=0.0045.***; PC2, F=29.19, p=4.84 x 10^{-7}.***).

Finally, the NE clade, *Polythore procera,* PCA resumes a 89% of the variation in PC1, the PC2 explains 5%. Almost all the landmarks contributed to PC1 (Fig 5). The MANOVA for the PC1 shows significance by sex (PC1, F=155.01, p=2.2 x 10^{-16}.***, Fig. 5, Table 2.), but no by population (F=155.01, p=2.2 x 10^{-16}.***, Fig. 5). We lack wings of the Colombian populations of *Polythore gigantea* (W Andes clade), so we only show the analysis for Ecuadorian individuals. In this case, there were only two PC that explained a total of 93% of the variation, and as in the NE clade the MANOVA only shows significance by sex in PC1 (PC1, F=274.9, p=4.7 x 10^{-8}.***, Fig. 5, Table 2).

DISCUSSION

Our phylogenetic reconstructions are consistent with the previous phylogenetic hypothesis for the family (Sanchez et al. 2018). The new populations collected in the Colombian Amazon basin and the Andes are related to the expected previously sampled in the same species geographic region (Fig. 1, Supplementary Figure 1., Sanchez et al 2018). However, the morphospecies, *Polythore beata,* collected in "Leticia" and "La Pedrera" seems to be two independent lineages. Our tree shows that *Polythore mutata* is sister to "La Pedrera", and "Leticia" is sister to both (Fig.2B). We also see this pattern in the haplotype's networks for all three loci (Fig. 3B, Supplementary Figure 2). Consistently, all these population all well differentiated (Fig. 4). Moreover, the differences between these taxa in the HW suggest there are independent entities (Fig. 5). In addition, the lineal map geographic distance between these two populations (i.e. "Pedrera" and "RNNP") is of 340.49 km, and they both are separated by two big rivers, Amazon

and Caqueta, that will merge down river in the Parana Arapu connection ~501.09 km from "La Pedrera" and ~567.29 km from "Leticia". The latter suggests that vicariance is important in the differentiation of these lineages.

In contrast with the Amazon, the Andean clades (W, SE, NE) populations have less structure. However, they have more haplotypic diversity. This result is consistent with a pattern of recent diversification in the Andes. Furthermore, different morphospecies, *P. neopicta* - *P. victoria*, showed no genetic differentiation in all but one population (Santa cruz, where only *P. victoria* was found). Although, Pozuzo (where, both *P. neopicta and P. victoria* coexist) populations are 32.2 km apart from Santa Cruz, these populations are separated by altitude (200 m) and the Panao river, which could be barriers to gene flow. Moreover, these two morphospecies had shared haplotypes in Pozuzo populations. In this locality both morphospecies are differentiated by the shape of HW color pattern. We suggest that this phenotypic divergence would be maintained by natural selection and sexual selection acting on color pattern variation as it has been demonstrated previously on butterflies (Jiggins, 2008).

The paraphyletic signal observed in *P. procera* suggest independent convergence in the shape of the color pattern. The high structure observed between Colombia and Ecuador, indicates that *P. procera* from Ecuador is an independent lineage and could be a cryptic species. In both cases, batesian mimicry with the butterfly *Greta andromica* are involved in maintaining the monomorphism in these populations. Thus, the same adaptive peak was colonized twice by the comimic damselfly.

Our data suggest that the morphospecies of *Polythore* in distinct geographic regions were formed by different processes. while Amazon lineages were shaped by vicariance associated to rivers and geographic distance without gene flow, Andean morphospecies showed a pattern of recent diversification promoted by dispersion. In the latter, the watershed system could allow divergence in presence of gene flow. However, a genomic scope awaits to be implemented in order to test the robustness of the mtDNA pattern and shows the real effect of dispersion and gene flow that shape *Polythore* lineages.

Finally, there is evidence that part of *Polythore* diversification is driven by the shape of color pattern variation. Mimicry selection is involved in monomorphic mimic rings with ithomiine butterflies (Outomuro, Ángel-Giraldo, Corral-Lopez, & Realpe, 2016) and could be also involved in the establishment polymorphic morphospecies as has been demonstrated in other insects such as butterflies (Jiggins, 2008). However, predation experiments are needed to validate this scenario. Also, the dimorphic phenotypes between sexes implies that sexual selection could be related to generate and keep polymorphisms in this genus.

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Tables

Table 1. Genetic diversity indexes for all tested *Polythore* populations within geographical clades for all the mtDNA gene fragments (16S, COI, ND1). ***h****:* number of haplotypes, ND: nucleotide diversity (**π**) and theta(pi) (**θπ**)

	16S				**COI**				**NDI**			
Clade	**Population**	***h***	**ND**	**Theta(pi)**	**Population**	***h***	**ND**	**Theta(pi)**	**Population**	***h***	**ND**	**Theta(pi)**
Amazon												
	Tiputini	4	0.021	7.051	*Tiputini*	3	0.003	2.076	*Pedrera*	1	0.013	NA
	Iquitos	1	NA	NA	*Iquitos*	1	NA	NA	*Tiputini*	4	NA	6.471
	Pedrera	2	0.001	0.500	*Pedrera*	2	NA	0.250	*Leticia*	1	NA	NA
	Rnnp	2	0.065	22.000	*Rnnp*	2	0.001	1.000				
NW												
	Mindo	2	0.001	0.476	*Mindo*	3	0.001	0.933	*Mindo*	1	NA	NA
	La Miel	1	NA	NA	*La Miel*	1	NA	NA	*Cotopaxi*	1	NA	NA
	Cotopaxi	1	NA	NA	*La Doctora*	3	0.006	4.473	*La Miel*	1	NA	NA
	La Doctora	1	NA	NA	*Copacabana*	1	NA	NA	*La Doctora*	1	NA	NA
SE												
	Pozuzo1	2	0.019	6.400	*Pozuzo1*	1	0.023	17.812	*Pozuzo1*	9	0.010	4.737
	Pozuzo2	1	NA	NA	*Pozuzo2*	5	0.006	4.911	*Pozuzo2*	2	0.001	0.264
	Pozuzo3	1	NA	NA	*Pozuzo3*	1	NA	NA	*Pozuzo3*	1	NA	NA
	Sta_Cruz	1	NA	NA	*Sta_Cruz*	1	NA	NA	*Sta_Cruz*	2	0.001	0.667
NE												
	La Catira	2	0.002	0.750	*La Catira*	2	NA	0.133	*La Catira*	3	0.012	5.676
	Chirajara	2	0.008	2.637	*Chirajara*	7	0.015	11.883	*Chirajara*	3	0.012	5.882
	Cementerio	1	NA	NA	*Santa Maria*	2	0.034	26.500	*Bavaria*	1	NA	NA
	Cubarral	3	0.007	2.467	*Rionegro*	3	0.007	5.333	*Cubarral*	3	0.015	7.200
	Bavaria	1	NA	NA	*Cementerio*	4	0.001	1.154	*Cementerio*	1	NA	NA
	Rionegro	2	0.004	1.333	*Bavaria*	5	0.002	1.385	*RioNegro*	2	0.008	4.000

	Santa Maria	2	0.037	12.500		*Santa Maria*	1	NA	NA

Table 2. MANOVA for the shape PC variables for both FW and HW for each geographic clade, tested by sex, species, and additionally by populations for *P. procera* only. (*) significative values indicating statistical differences between the tested clusters.

		Fore Wing					
Clade	**Group**	**Component**	**DF**	**Sumsq**	**Mean**	**F value**	**Pr (>F)**
Amazon	Sex	**PC1(63%)**	1	0.02339	0.023385	0.4286	0.5179
		Residuals	29	1.58242	0.054566		
		PC2(20%)	1	0.00003	3.23E-05	0.0019	0.9659
		Residuals	29	0.50355	0.017364		
	Species	**PC1(63%)**	2	1.56044	0.78022	481.56	**2.20E-16***
		Residuals	28	0.04537	0.00162		
		PC2(20%)	2	0.16043	0.080216	6.5454	**0.004654***
		Residuals	28	0.34315	0.012255		
Neopicta-victoria	Sex	**PC1(49%)**	1	0.37539	0.37539	13.147	**0.000519***
		Residuals	76	2.17011	0.02855		
		PC2(23%)	1	0.7296	0.7296	120.03	**2.20E-16***
		Residuals	76	0.46197	0.00608		
	Species	**PC1(49%)**	1	0.19455	0.194551	6.2893	**0.01428***
		Residuals	76	2.35096	0.030934		
		PC2(23%)	1	0.06151	0.061514	4.137	**0.04545***
		Residuals	76	1.13006	0.014869		
Procera	Sex	**PC1 (89%)**	1	6.3717	6.3717	155.01	**2.20E-16***
		Residuals	64	2.6308	0.0411		
		PC2 (5%)	1	0.03202	0.032023	3.8336	0.0546
		Residuals	64	0.53461	0.008353		
	Populations	**PC1(89%)**	5	0.8261	0.16523	1.2125	0.3144
		Residuals	60	8.1763	0.13627		
Gigantea	Sex	**PC1 (78%)**	1	1.05899	1.05899	274.91	**4.71E-08***
		Residuals	9	0.03467	0.00385		
		PC2 (15%)	1	0.0087	0.0087	0.2795	0.6098
		Residuals	9	0.28017	0.03113		
	Hind Wing						
Amazon	Sex	**PC1(63%)**	1	0.27966	0.279663	4.8913	**0.03503***
		Residuals	29	1.65808	0.057175		
		PC2(20%)	1	0.25926	0.259262	10.709	**0.002755***
		Residuals	29	0.70207	0.024209		
	Species	**PC1(63%)**	2	1.19705	0.59853	22.626	**1.42E-06***
		Residuals	28	0.74069	0.02645		
		PC2(20%)	2	0.3049	0.152451	6.5028	**0.004791***
		Residuals	28	0.65643	0.023444		

Neopicta-victoria	Sex	**PC1(49%)**	1	1.8173	1.81731	58.365	**1.72E-11***
		Residuals	95	2.958	0.03114		
		PC2(23%)	1	0.08016	0.080155	3.1216	0.08047
		Residuals	95	2.43939	0.025678		
	Species	**PC1(49%)**	1	0.3956	0.39561	8.5812	**0.004252***
		Residuals	95	4.3797	0.0461		
		PC2(23%)	1	0.59224	0.59224	29.192	**4.84E-07***
		Residuals	95	1.92731	0.02029		

Figures

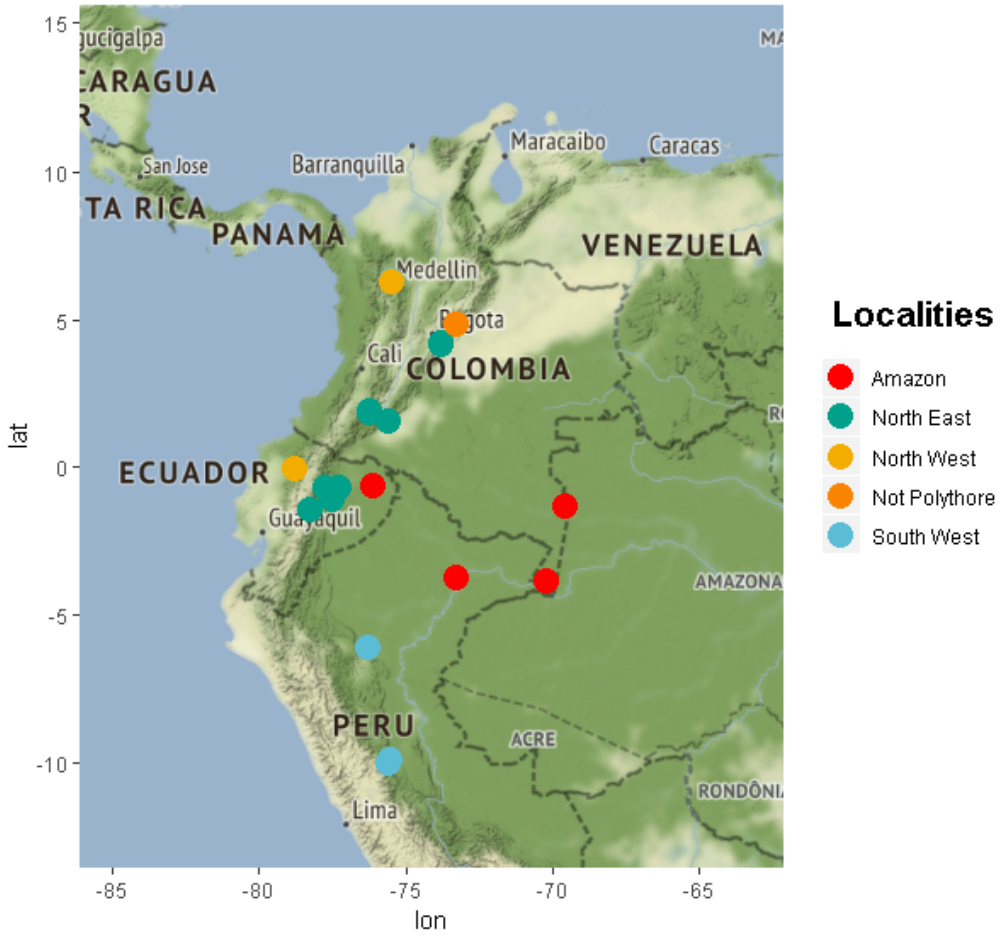


Figure 1. **Taxon sampling across South America**. All *Polythore* specimens are grouped by geographic clades as described by (Sánchez et al., 2018).

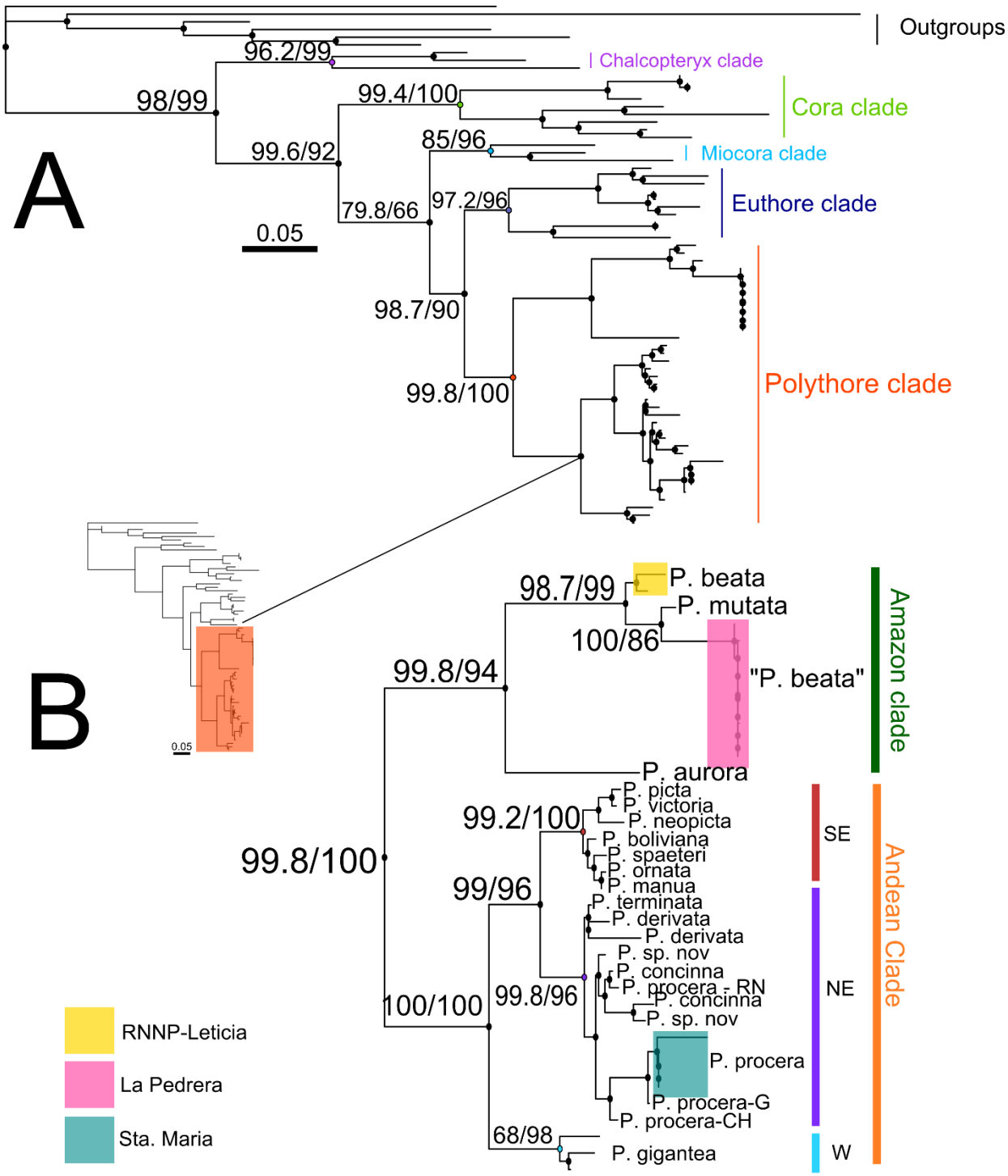


Figure 2. Phylogenetic reconstructions for the family Polythoridae and genus *Polythore*. A. Best recovered maximum likelihood phylogram of the Polythoridae family. UFBootstrap and SH-Lrt supports are presented above the branches. **B.** Phylogram of *Polythore* indicating the Amazon and Andean geographic clades. Sampled individuals for the RNNP, La Pedrera and Santa Maria are highlighted in colors, as well as well supported geographic clades, SE: South East; NE:North East; and W: for West).

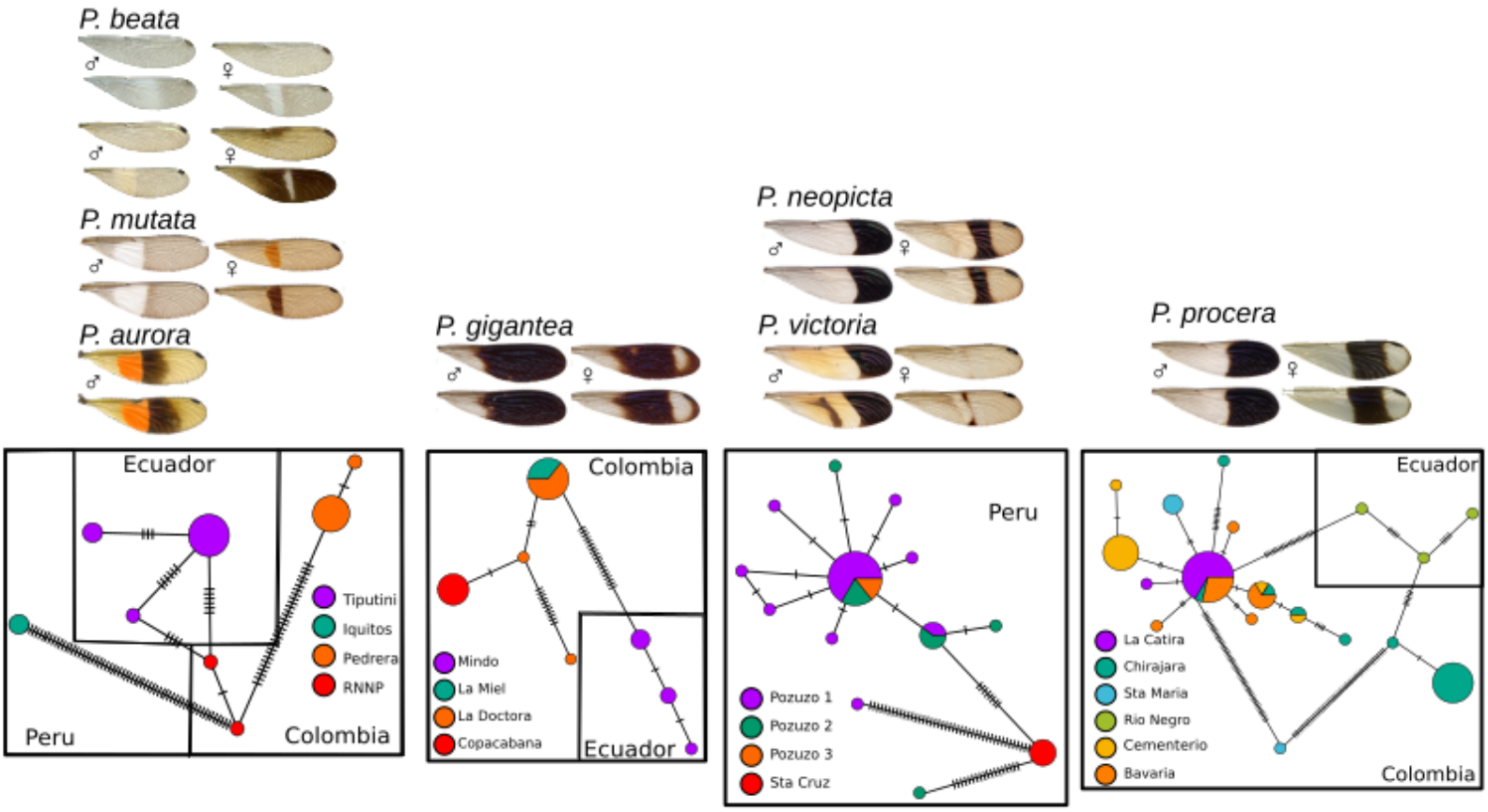


Figure 3. Haplotype networks and wing diversity. A. Wings of *Polythore* morphospecies and, B. COI minimum spanning networks for each geographical clade indicating populations by color and the country where each population belongs to.

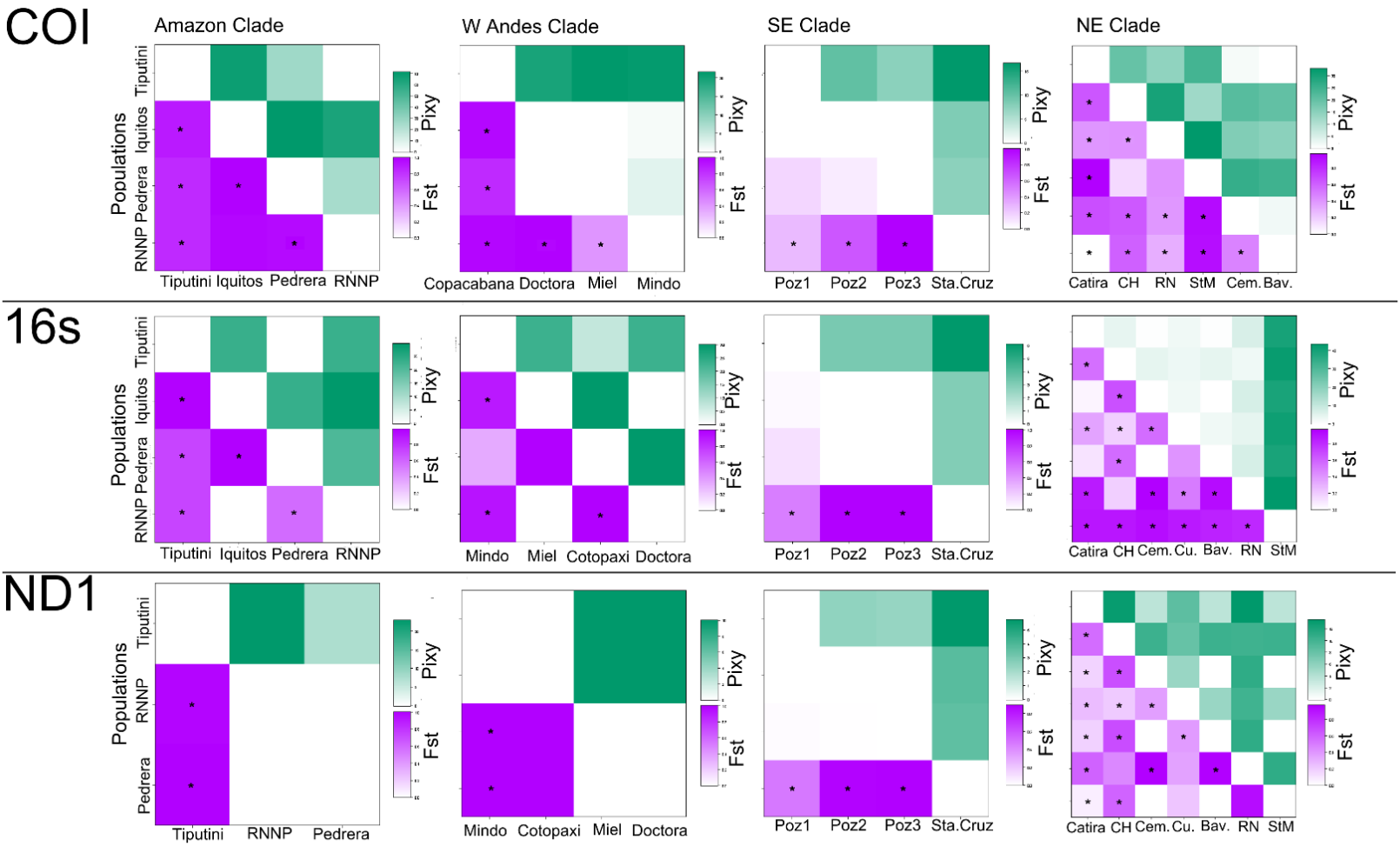


Figure 4. Fst and pairwise distances among all populations of *Polythore* for all three loci. (*) mean significant values.

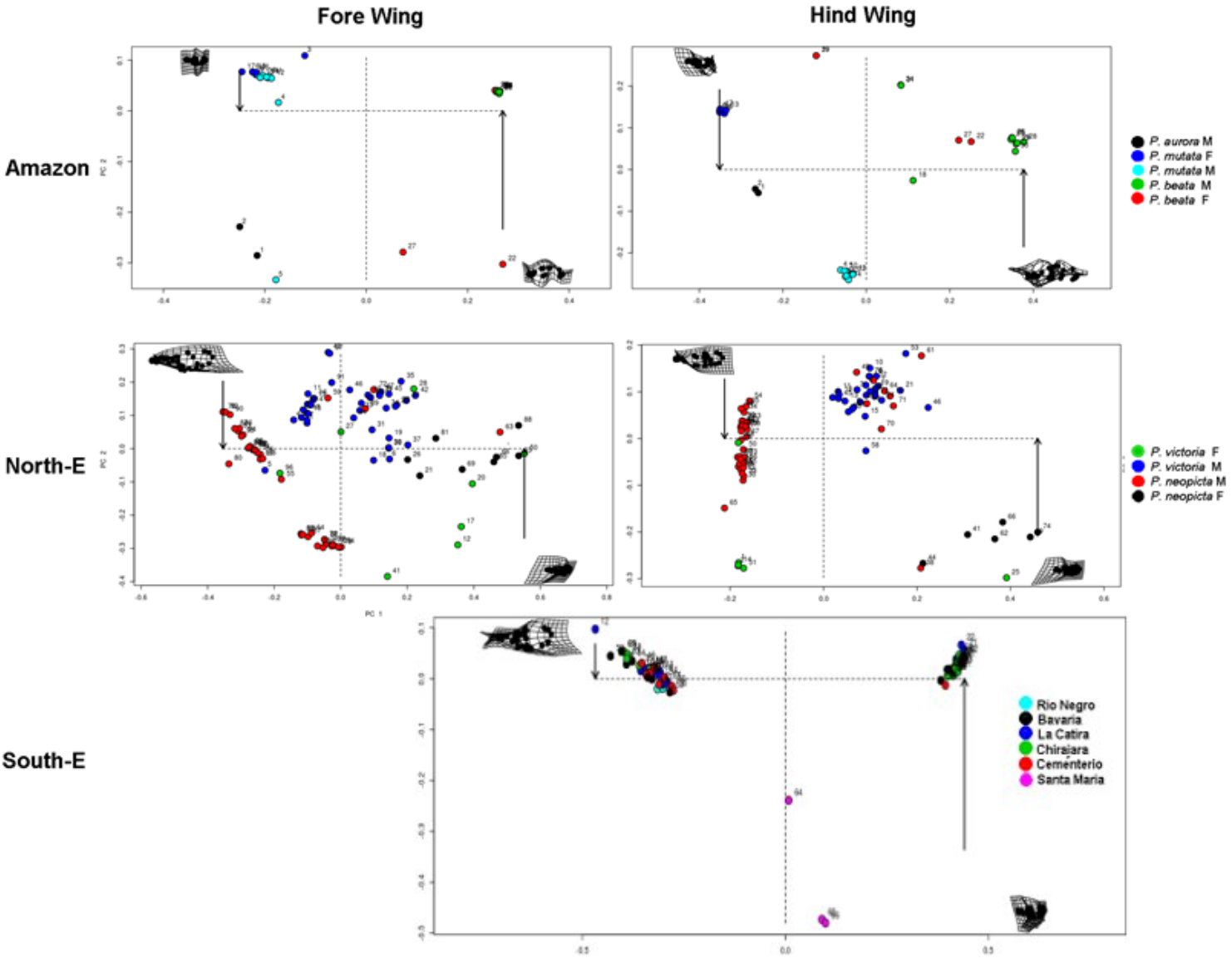


Figure 5. Morphometric analyses of the wing color pattern shape for the target species for each clade.

Supplementary Material.

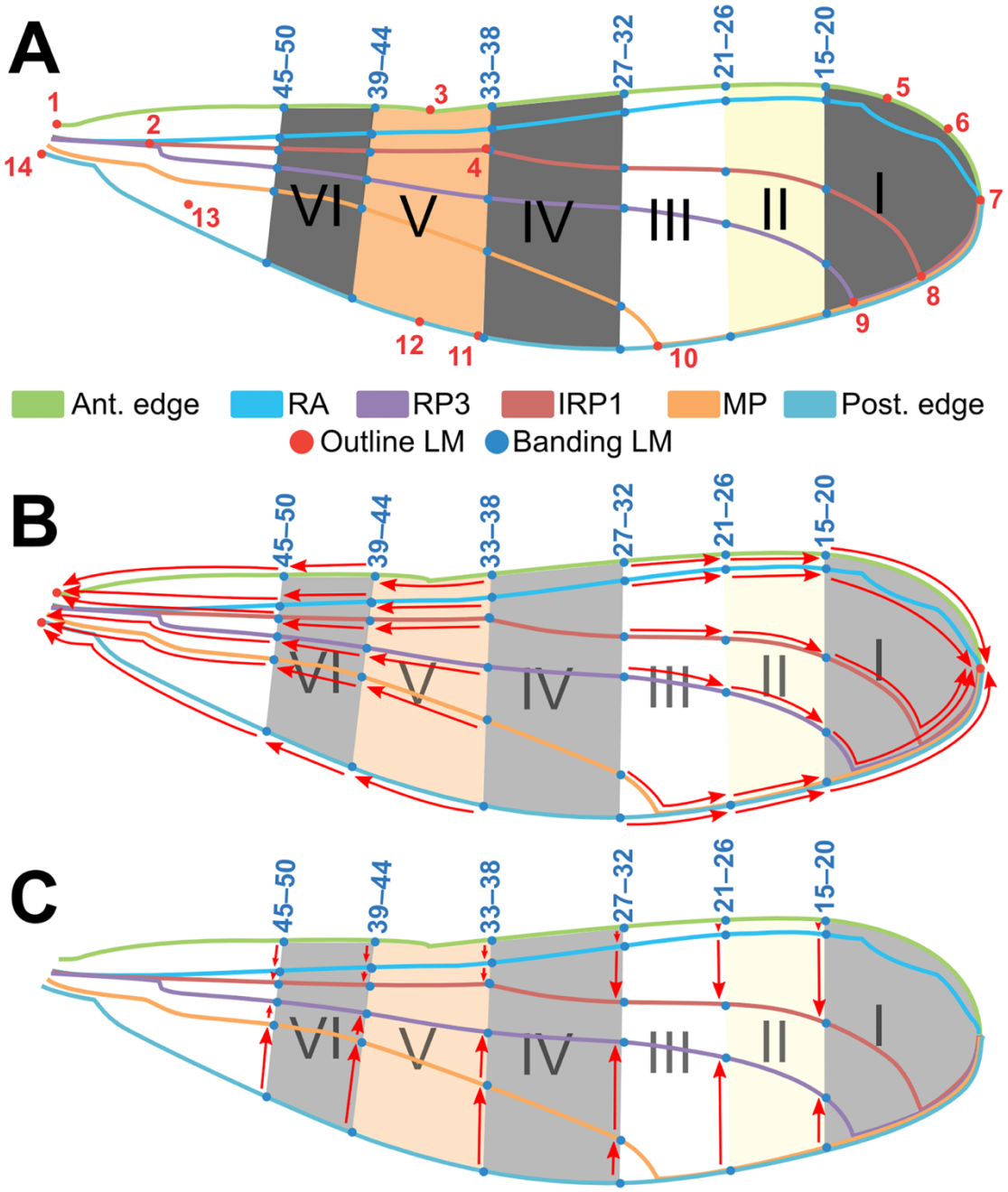
Supplementary Appendix 1.

PCR conditions and protocols.

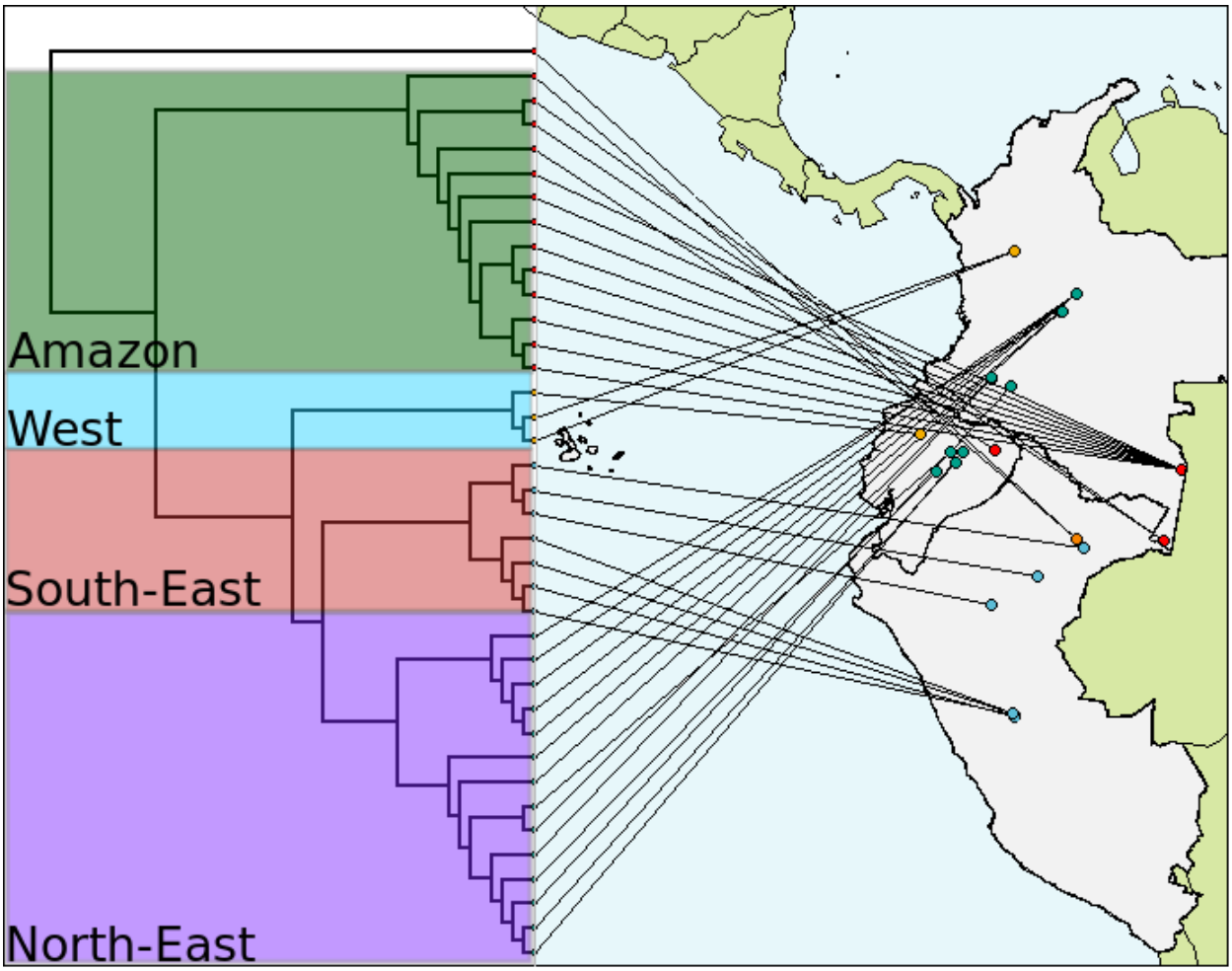
All PCR reactions were 25ul final volume reactions containing 1 to 2 uL of DNA and Green GoTaq Master Mix (PROMEGA, 2007) if not by components of 1X Buffer, 2 MM $MgCL_2$, dNTPs, 0.5 mM of each primer and 1.25u of GoTaq DNA Polymerase. PCR thermal profiles were for each fragment: 1) COI: 94°C for 2min, followed by 15 cycles of 94°C for 45s, 48°C for 45s, 72°C for 45s, and then 35 cycles of 94°C for 30s, 52°C for 30s, 72°C for 45s and a final cycle of 72°C for 5 min; 2) 16s: 94°C for 2 min, followed by 15 cycles of 94°C for 45s, 48°C for 45s, 72°C for 45s, and then 35 cycles of 94°C for 30s, 49°C for 45s, and finally 72°C for 4 min. At last; 3) NDI: 95°C for 2 min followed by 10 cycles of 95°C for 30s, 49°C for 30s, 72°C for 45s, and then 25 cycles of 95°C for 30s, 50°C for 30s, 72°C for 45s and one last cycle at 72°C for 4 min.

Supplementary Appendix 2.

Landmarking protocol based on Sanchez Herrera et al. 2015.



Supplementary Fig 1. Map and taxon sampling across the *Polythore* clades.



Supplementary Figure 2. Minimum spanning-networks for 16S and ND1, for all *Polythore* clades.