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Genetic variation of *Limnophora marginata* Stein, 1904 (Diptera: Muscidae), associated with an altitudinal gradient in Pedro Moncayo Canton, Pichincha, Ecuador

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Abstract. *Aguilar KEV, Vivas AYS, López CA. 2023. Genetic variation of* Limnophora marginata *Stein, 1904 (Diptera: Muscidae), associated with an altitudinal gradient in Pedro Moncayo Canton, Pichincha, Ecuador. Biodiversitas 24: 269-275.* Analyzing heterogeneity in insect populations is relevant to understanding its biology. Thus, we analyzed the genetic variability of two populations of "hunter-flies", *Limnophora marginata* Stein, 1904 associated with an altitudinal gradient in the Tocachi and Jerusalem localities, in the Pedro Moncayo Canton, Ecuador, using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the mitochondrial Cytochrome oxidase I gene. Thirty alleles were detected from analysis with five restriction enzymes; the percentage of polymorphic loci was higher in Tocachi (96.67%) than in Jerusalem (80%). Haplotypes were generated with the corresponding alleles, resulting in a different haplotype for each analyzed individual. Several estimators were used to characterize the population structure. The Analysis of Molecular Variance (AMOVA) evidenced the existence of higher intra-population variability (94%) than inter-population (6%), showing similar information to that found through haplotype analysis. The Fst fixation index indicated that there is no genetic difference between populations (0.056, $P<0.05$). Nei's genetic distance and Nei's similarity index indicated that *L. marginata* populations did not show differences between populations. From the results we deduce that the evolutionary force that may be modelling the existent genetic variability may be gene flow, thus establishing a low inter-population genetic structure. The mitochondrial DNA Cytochrome oxidase I gene was an advantageous molecular marker to obtain results that established intra and inter population variability. The characteristics of the mitochondrial DNA were useful to successfully apply the PCR-RFLP technique. Our findings highlight the existence of ecosystem connectivity between *L. marginata* populations despite altitudinal and habitat differences.

Key words: COI gene, genetic variability, hunter—flies, molecular markers, PCR-RFLP

INTRODUCTION

The Muscidae family is located along all the biogeographic regions that do not present arid habits (Achint and Singh 2021). In higher altitudes and latitudes, they include a great number of individuals and species, exhibiting a total of 846 species for the neotropical region (Fogaça and de Carvalho 2015). The Coenosiinae subfamily shows the greater number of species in the family, since it represents 40% of all the muscids (De Carvalho 2002). A main characteristic in this subfamily is the preference of the larvae for water bodies. Its diet consists of decomposing vegetation (Jassem et al. 2022), small insects, such as aphids, flies from the Agromyzidae and Chloropidae families, but mainly mosquitos from the Simulidae and Culicidae families (Pont et al. 2012). Coenosiinae is divided in the tribes: Coenosiini and Limnophoriini (Pont and Ivković 2013).

The Limnophoriini tribe is cosmopolitan, generally associated with aquatic environments, although they have been found in dry ones (Jassem et al. 2022). The genus *Limnophora* is found in every region in the world; Xue et al. (2012), Pont et al. (2012), Pont and Ivković (2013) and Couri and Pont (2017) indicate its wide distribution, occupying habitats in Palearctic, Nearctic, Neotropical, Ethiopic, Indian, and Australian regions. However, in North America there are fewer species and in New Zealand there is no record for it (Pont et al. 2012), there are approximately 350 species inside this genus in the world (Pont et al. 2012). In Ecuador, nine species have been registered (Fogaça and De Carvalho 2015). *Limnophora* larvae are aquatic and semi-aquatic (Pont and Ivković 2013), they opt for being close to algae and moss, since several flies oviposit in these substrates, giving them food and shelter (Glime 2021). Generally, they feed from individuals from the Chironimidae and Simulidae families (Ivković and Pont 2016). *Limnophora* has been registered in adult phase near running water in mountain zones, such as rivers and streams (Pont and Ivković 2013).

Several species of *Limnophora* have been evaluated, in Argentina for example, through the synantrophy index, giving as a result that 100% of the collected individuals were found in rural zones (Patitucci 2010). While in Ecuador research conducted by Blacio et al. (2020), revealed that *Limnophora marginata* Stein, 1904 shows a preference for urban areas, with a high index of synantrophy. In addition, the species is distributed in Argentina, Brazil, Colombia, and Ecuador (Fogaça and de Carvalho 2015).

Genetic variation is understood as the variations in the genome that are heritable. Generally, variability between populations is generated through several factors such as:

geographical isolation, ecological isolation, and historical processes (Wang et al. 2017); and it is kept through natural selection, migration, mutation, genetic drift, or changes in population size. It is also linked to the allelic frequencies that vary among generations (Charlesworth 2015), establishing the proportion of a particular allele in a population throughout generations. Genetic variation analyses are important to understand the genetic structure of natural populations and how different environmental factors, such as altitude, can influence the population's diversity. Analyzing heterogeneity in insect populations is relevant to understanding its biology (Leffer et al. 2012).

Molecular markers facilitate the analysis of genetic variability, allowing to identify polymorphisms, indicating sites close to a gene that are found in a specific locus. On other occasions genes are used as molecular markers themselves. Several molecular markers such as the mitochondrial DNA ($_{\text{MT}}$DNA) are used to evaluate the genetic structure of populations. Mitochondrial DNA is one of the most used and suggested, due to its maternal inheritance, null genetic recombination, high copy number and easy isolation (Achint and Singh 2021). From $_{\text{MT}}$DNA, the cytochrome C oxidase I (COI) gene shows a high rate of evolution and variability, due to its mutation rate (Khullar et al. 2016). It is highly used because the regions that encode the protein are highly conserved, but variable in their sequence (Achint and Singh 2021). COI has been found to successfully differentiate between species being useful for identification and phylogeny. One of the techniques for analyzing genetic variability of $_{\text{MT}}$DNA is polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP), using restriction enzymes that cut specific sites in the DNA sequence to detect polymorphisms (Alam et al. 2022). However, such analyses of the genetic variability of flies from the *Limnophora* genus, associated with changes in altitude have not been conducted in Ecuador. The main aim of the present study was to compare the genetic variation between *L. marginata* populations located in two different locations in the Pedro Moncayo Canton, in the Andean Region of northern Ecuador.

MATERIALS AND METHODS

Study area

The population of this study includes 526 *L. marginata* individuals for the Tocachi parish in Evergreen High Montane Forest (-78.290500 W 0.048400 N) and 32 for the Malchingui parish in Dry Forest (-78.358324 W-0.000693 S), located at the Pedro Moncayo Canton of the Pichincha Province, Ecuador (Figure 1). Samples were collected through necrotraps as established in Ruiz et al. (2021). The sample was selected in coordination with the parallel morphogeometrics project (Ruiz et al. 2021). Due to the difference in population size between localities and the need to standardize the protocol and preserve voucher samples, twenty-six female individuals of each location were used. For the next procedures the entirety of the individual was used minus its wings that were analyzed for morphogeometric markers (Ruiz et al. 2021).

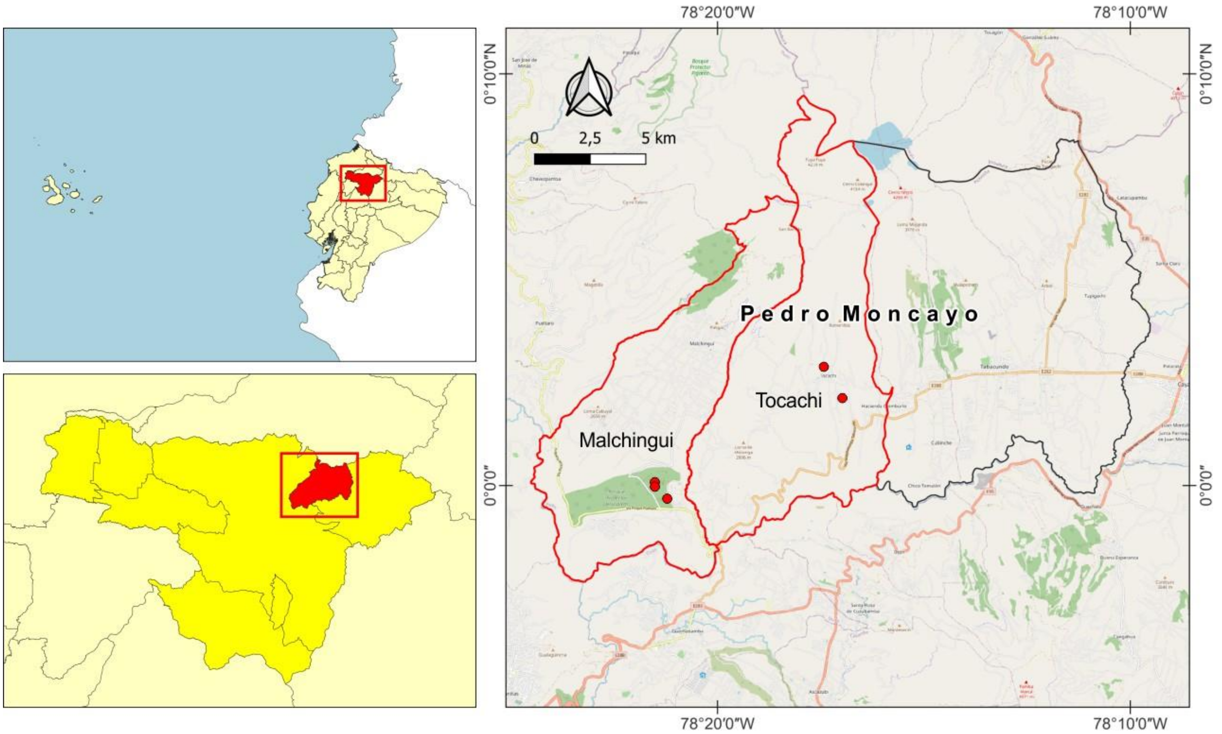

Figure 1. Geographical location of the two studied populations in Tocachi and Malchingui, Ecuador. The specified points indicate the coordinates of the subsampling locations in each parish

Sampling and DNA extraction

DNA extraction was based in the protocol proposed by Sunnucks and Hales (1996) and Honda (2008), with some modifications. Each individual separately was washed with distilled water, then crushed with the lysis buffer (Tris 50 mM, pH 7.5, NaCl 400 mM, Ethylene diamine tetra-acetic acid (EDTA) 20 mM, sodium dodecyl sulfate (SDS) 10%), and 20 µL of Proteinase K abm® were added. Samples were then incubated at 56°C for 21 hours in a water bath WH-4C Medical expo ZENITHLAB ®. After incubation, 170 µL of NaCl 5 M were added and then the samples were centrifuged at 13.500 rpm for 10 minutes in HERMLE Z 207M centrifuge. Supernatant was collected carefully and then 400 µL of ethanol 96% was added to precipitate the DNA. Finally, the pellet was obtained by centrifuging the sample for 1 minute at 13.500 rpm. The pellet was washed two times with ethanol 70%. The obtained DNA was resuspended in 50 µL of nuclease free HPLC water and preserved at-21°C.

PCR-RFLP analysis

For amplification of a fragment of the COI gen we used the following protocol: 7.5 µL nuclease free water, 2.5 µL PCR Buffer-Mg 10X Invitrogen® Thermo Fisher Scientific, 2 µL of the forward primer 5'–GGTCWACWAATCATAAAGATATTGG-3', 2 µL of the reverse primer 5'-RAAACTTCWGGRTGWCCAAARAATCA-3', described by Nelson et al. (2007), 1,5 µL of MgCl2 50Mm Invitrogen® Thermo Fisher Scientific, 1 µL de dNTPs abm®, 1 µL de Taq Platinum 2U Invitrogen® Thermo Fisher Scientific and 7,5 µL of DNA, for a final volume of 25 µL, following the suppliers recommendation, Invitrogen®.

PCR was conducted under the following time and temperature conditions: initial denaturation at 95°C for 5 min for one cycle, denaturation at 94°C for 1 minute, annealing at 49°C for 1 minute, extension at 72°C for 1 minute during 35 cycles and a final extension at 72°C for 4 minutes. Samples were kept at-21°C until used for the restriction analysis. The PCR products were digested with the following restriction enzymes Anza™: 111 Xapl (20U/µL), 77 Dral (10U/µL), 42 Rsal (5U/µL), 65 Mspl (20U/µL) and 76 Vspl (10U/µL) Invitrogen® 38 of Thermo Fisher Scientific. Restriction protocol was prepared as follows: 16 µL of nuclease free water, 2 µL Red Buffer Anza, 1 µL of the obtained PCR product and 1ul of the Anza respective enzyme, for a final volume of 20 µL. Samples were incubated at 80°C for 15 minutes in the Mini Amp Plus™ Thermo Fisher Scientific thermocycler and then refrigerated. PCR and PCR-RFLP results were observed through 1,5% agarose gel electrophoresis and TBE 1X, for 40 minutes at 300V.

Data base elaboration and analyses

Images of the gel results of the enzymatic digestion were obtained through the Microtek software and were edited with the Slate filer of Microsoft Office. Each image was classified according to the population of *L. marginata* Stein, either Tocachi or Jerusalem, and then further subclassified according to each enzyme. The size of the fragments was estimated in comparison with the used ladder Biogena© Cat. No: G106 1Kb, to differentiate each allele. The found alleles were registered in a Microsoft Excel matrix as binary data, including 1 when the corresponding band was present and 0 when it was absent. Later, the data were inserted in the GenAlex complement following the instructions in the software's manual (Peakall and Smouse 2006). The informatic packages used for analyzing the genetic variation between the two populations were Arlequin 3.5 (Excoffier and Lischer 2010), Textpad and the GenAlex complement in Microsoft Excel (Peakall and Smouse 2006). Population structure was assessed by calculating Nei's Genetic Distance, F_{ST} Wright statistic and Analysis of Molecular Variance (AMOVA).

RESULTS AND DISCUSSION

Digestion of COI amplified region with the five enzymes generated 30 alleles in *L. marginata* Tocachi population, 25 of which are shared with the Jerusalem population. Each allele was established due to its size in base pairs. We have established the patterns observed for each enzyme (Table 1). This information was later used to develop the corresponding haplotypes (Table 2).

Each analyzed individual was found to have a different haplotype, indicating that there is a high intrapopulation diversity in the studied populations of *L. marginata*. Most of the identified alleles are shared in and between the populations, this is reflected in the established haplotypes. No haplotype was shared completely in or between the populations. This event is possibly because of the presence of variable sites in the COI gene as a marker (Alam et al. 2022), which could explain the registered genetic variability.

The analyzed genetic parameters allowed us to compare the genetic variability between the two localities (Table 3). The percentage of polymorphic loci in the Tocachi population was of 96.67%, while in the Jerusalem one it was 80%, pointing out the intrapopulation genetic variability, which can be caused by dispersion. There are no geographic barriers between the studied locations, on the contrary, there is a bio-corridor Pisque-Mojanda-San Pablo (Ordoñez and Domínguez 2015) between populations, which facilitates the connectivity between these two populations. These results are comparable with Tiroesele et al. (2014) who when analyzing the genetic variability and population structure of *Cerotoma trifurcata* Forster, 1771, found it to be due to an elevated gene flow because of the lack of geographic barriers separating the different populations. In addition, Moreira and Soto-Vivas (2019) established the distribution of the species between 2228 and 3129 masl, maintaining the variability along the altitudinal gradient.

An AMOVA analysis was conducted using GenAlEx (Peakall and Smouse 2006) to estimate the populations' structure. The AMOVA found a 6% variance between populations and 94% within populations (p value 0.057), indicating a low genetic variability interpopulation and a high intrapopulation genetic variability. Likewise, the Genetic Nei's distance found a value of 0.041 (Table 4.) highlighting a minimal interpopulation difference and a high similitude (Manzo-Sánchez et al. 2017).

Calculations of the Fst Wright statistic in Arlequin 3.5 (Excoffier and Lischer 2010) to estimate the interpopulation differentiation among the analyzed populations casted a value of 0.05676 (p value = 0.00293 ± 0.016) confirming the results from the mentioned analyses, indicating that there is no differentiation between the populations under study. These results suggest that genetic flow exists between populations. Similar results were found by Padwal et al. (2021) revealing a 93.13% variation within populations and 6.87% among populations, with a genetic distance of 0.004.

On the contrary, research conducted by Seabra et al. (2015) in characterizing the genetic population structure of Ceonosia attenuata Stein worldwide through $_{MT}$DNA; in contrast with the present study, found high similarity between haplotypes. At the same time variation was found to be higher among groups than inside them. These differences with our study and that of Padwal et al. (2021) may be due to a higher sampling per locality in our study, while the mentioned study used 150 samples worldwide, considering 17 countries. Considering the differences through an altitudinal gradient, Demirci et al. (2012) proposes that altitude was not a determinant factor in the population genetic structure of *Culex theileri* Theobald, 1903 (Diptera: Culicidae) since genetic flow oversees maintaining connectivity between populations.

These aspects correspond to the results found in this investigation, since the altitudinal gradient did not influence the interpopulation genetic variability. Our parallel project analyzing morphological differences with geometric morphometric tools (Ruiz et al. 2021) concluded that the found diversity was probably due to the plasticity of the species. Encompassing such analyses with molecular markers results in a more comprehensive view of the populations dynamics and allows us to better understand changes in population.

Table 1. Diagnostic restriction patterns obtained by PCR-RFLP of the COI gene of *L. marginata*. Capital letters indicate polymorphic restriction patterns found for each enzyme

	COI			COI	
Restriction enzyme	**Pattern**	**Fragments (bp)**	**Restriction enzyme**	**Pattern**	**Fragments (bp)**
Xapl	A	1000, 800, 600, 500, 200, 100	Mspl	A	1500, 700, 500, 200,100
	B	1000, 800, 500, 200, 100		B	2000, 1500, 200, 100
	C	1000, 900,800, 500, 200		C	1500, 200, 100, <100
	D	1000, 500, 200, 100		D	2000, 1500, 200
	E	1000, 600, 500, 200		E	1500, 200, 100
	F	1000, 800, 500, 200		F	1500, 200
	G	1000, 500, 200		G	1500, 100
	H	500, 200, 100		H	2000, 100
	I	900, 500		I	1500
	J	1000		J	2000, 1500, 100
	K	1000, 500, 200		K	2000
	L	1000, 500		L	2000, 1500
	M	100			
Rsal	A	1000, 700, 400, 300, 200, 100	Vspl	A	1500, 1000, 700, 500, 200
	B	1000, 400, 300, 200, 100		B	1500, 700, 500, 400, 200
	C	1000, 700, 400, 300,200		C	1500, 1000, 500, 400, 200
	D	1000, 400, 300, 200		D	1500, 500, 400, 200, 100
	E	1000, 400, 300, 100		E	1500, 500, 400, 100
	F	1000, 400, 300		F	1000, 500, 400, 200
	G	1000, 400, 100		G	1500, 500, 400, 200
	H	1000, 100		H	1000, 700, 500, 200
	I	1000, 400		I	1000, 500, 400
	J	1000		J	1500, 500, 400
	K	1000,700, 400, 100		K	1500, 500, 200
	L	700, 200		L	500, 400, 100
				M	1500, 500
				N	500
Dral	A	700, 600, 100		O	1000
	B	700, 600		P	1500
	C	600, 100		Q	200
	D	600		R	1500, 500, 200, 100
				S	1500, 700, 500, 200
				T	1500, 100
				U	1500, 400
				V	500, 100
				W	500, 200
				X	100

Table 2. Composite haplotypes based on the restriction patterns obtained for each enzyme

Haplotype Tocachi Population	COI Xapl	Rsal	Dral	Mspl	Vspl	Haplotype Jerusalem Population	COI Xapl	Rsal	Dral	Mspl	Vspl
L. marginata_1	H	H	C	I	I	*L. marginata_1*	F	B	A	I	K
L. marginata_2	D	E	A	I	E	*L. marginata_2*	D	G	A	I	-
L. marginata_3	G	G	A	I	L	*L. marginata_3*	K	E	A	I	I
L. marginata_4	-	J	A	-	A	*L. marginata_4*	G	E	A	E	M
L. marginata_5	G	B	A	A	F	*L. marginata_5*	G	E	B	F	-
L. marginata_6	E	J	A	D	F	*L. marginata_6*	K	E	B	I	-
L. marginata_7	G	F	A	F	F	*L. marginata_7*	D	B	B	E	M
L. marginata_8	I	I	A	D	B	*L. marginata_8*	G	K	D	G	X
L. marginata_9	G	J	A	I	-	*L. marginata_9*	-	I	A	L	X
L. marginata_10	I	J	A	D	N	*L. marginata_10*	L	G	B	G	X
L. marginata_11	I	I	B	I	O	*L. marginata_11*	D	B	A	G	D
L. marginata_12	G	A	A	B	P	*L. marginata_12*	G	G	A	I	P
L. marginata_13	F		B	E	G	*L. marginata_13*	-	G	A	G	T
L. marginata_14	G	C	B	F	Q	*L. marginata_14*	L	I	B	I	P
L. marginata_15	J	B	B	G	M	*L. marginata_15*	G	B	A	I	J
L. marginata_16	-	-	B	I	C	*L. marginata_16*	G	D	A	I	G
L. marginata_17	G	B	B	H	J	*L. marginata_17*	M	I	A	F	G
L. marginata_18	B	B	B	E	J	*L. marginata_18*	L	E	A	F	M
L. marginata_19	F	F	B	F	B	*L. marginata_19*	B	E	B	F	R
L. marginata_20	B	E	B	E	G	*L. marginata_20*	D	E	A	E	P
L. marginata_21	A	D	A	C	D	*L. marginata_21*	-	J	B	E	U
L. marginata_22	C	J	B	F	A	*L. marginata_22*	-	E	A	L	V
L. marginata_23	G	J	A	F	J	*L. marginata_23*	-	E	B	I	W
L. marginata_24	F	F	A	F	K	*L. marginata_24*	-	J	A	K	U
L. marginata_25	D	F	B	F	K	*L. marginata_25*	-	J	B	G	S
L. marginata_26	H	I	B	I	H	*L. marginata_26*	-	L	A	J	X

Table 3. Genetic parameters: sample size (N), number of alleles (Na), Shannon-Weaver diversity index (I), haploid genetic diversity (h) and percentage of polymorphic loci (%P) per population for the COI MTDNA

Population		N	Na	I	He	h	%P
Tocachi	Media	26	1.967	0.461	0.31456	0.302	96.67%
	SE	0	0.033	0.037	0.16688	0.029	
Jerusalem	Media	26	1.633	0.400	0.27836	0.268	80%
	SE	0	0.140	0.050	0.20764	0.036	

SE: Standard error

Table 4. Nei genetic distance between Tocachi and Jerusalem (Ecuador) populations of *L. marginata*

	Tocachi	Jerusalem
Tocachi	0.000	
Jerusalem	0.041	0.000

The presence of two unique alleles in the Tocachi population is also important to mention, this could be explained by the taxonomic composition of Muscidae in the studied locations. The *L. marginata* was the most abundant species in Tocachi with 526 individuals and the second most abundant one with 32 individuals in Jerusalem. This difference in the number of individuals can possibly be associated with the specificity of the species for water bodies (Wotton and Merritt 1988; Patitucci 2010) characteristic of the Tocachi location (MAE 2013). In addition, both the unique alleles as well as the maximum values of the allelic frequencies of some alleles for the populations can be caused by the demographic history of the populations, these could have been influenced by genetic micro differentiation some time ago, due to the environmental characteristics.

The analyses of the different estimators of genetic variability inter and intrapopulation were useful since they allow the identification of an heterogenous population of *L. marginata* for both populations, Tocachi and Jerusalem, establishing a high intrapopulation genetic variation. The genetic diversity results are supported by the research conducted by Andrić et al. (2017) in populations of *Merodon ruficornis* Meigen, 1822 (Diptera: Syrphidae) in different localities. Its findings of the existence of variability were related with a wide distribution from the species without an influence of geographic distance. Our results also establish that the populations from both locations are highly similar and show a low genetic structure, suggesting that the studied populations are not isolated from one another. Considering this information, we can establish that characteristics found in the studied populations reflect an ecosystem connectivity, despite being found in different altitudes and ecosystems. As such, we determine that using COI as a molecular marker there is no interpopulation genetic differentiation found.

Studies of the genetic diversity of populations can describe the influence of environmental factors in Diptera populations, the existence of differentiation, the possible evolutionary forces shaping the genetic structure, and other characteristics; allowing us to better describe the biology of these species. In this species we found that there is genetic flow between the populations, which is the responsible evolutionary force for a low interpopulation genetic variability since it allows panmixia between populations and keeps them homogenous. The amount of genetic flow existent allows populations to evolve, whether it is dependent or independently from each other, stirring the possibilities of adaptation and posterior evolution in populations from the same species.

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