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Campus de São José do Rio Preto

Marjorie da Silva

Phylogeographic analysis in populations of *Protonectarina
sylveirae* (Saussure, 1854) (Hymenoptera, Vespidae,
Polistinae).

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Tese apresentada como parte dos requisitos para obtenção do título de Doutora em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de São José do Rio Preto.

Orientador: Prof. Dr. Fernando
Barbosa Noll

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*“Posso ouvir o vento passar
assistir à onda bater
mas o estrago que faz
a vida é curta pra ver”*

Rodrigo Amarante/Los Hermanos

RESUMO

Os processos históricos e ecológicos de diversificação da fauna têm sido amplamente estudados sob a luz da filogeografia, disciplina que lida com o arranjo espacial das linhagens genéticas. As vespas enxameadoras (Epiponini) como *Protonectarina sylveirae* (Saussure) são representantes comuns da fauna Neotropical. Esta espécie ocorre amplamente pela floresta Atlântica e apresenta uma distribuição peculiar dentro de Epiponini devido à sua ausência na região Amazônica. Apesar de muitos estudos apontarem para a existência de descontinuidades filogeográficas para diferentes organismos na floresta Atlântica, explicações sobre as causas de tais padrões permanecem inconclusivas. Este trabalho teve como objetivo investigar o padrão filogeográfico de *P. sylveirae*, com especial interesse na presença de estruturação genética e diferenciação morfológica, bem como nos eventos históricos que possam explicar o padrão filogeográfico observado. Os espécimes de *P. sylveirae* foram coletados ativamente em 13 áreas ao longo de sua distribuição para a extração, amplificação e sequenciamento dos genes mitocondriais 12S, 16S e COI. Foram realizadas análises de diversidade genética, demografia histórica, tempo de divergência e estruturação populacional. Identificou-se 22 haplótipos, uma forte estruturação genética foi encontrada pela análise de variância molecular (AMOVA) e a rede de haplótipos revelou 3 grupos de haplótipos, também corroborados pela AMOVA. As análises de demografia mostraram estabilidade populacional para. Encontrou-se diferenças na morfologia, corroborando parcialmente aquelas obtidas com os dados moleculares. A análise do tempo de divergência sugere que os eventos orogênicos do terciário e o processo de aridização das áreas de floresta que ocorreu a partir deste período influenciaram a diversificação e na atual configuração da distribuição das populações de *P. sylveirae*.

Palavras-chave: Filogeografia. Epiponini. Região Neotropical. mtDNA. Quaternário.

ABSTRACT

Ecological and historical processes of fauna diversification have been widely studied under the light of phylogeography, a discipline that deals with the spatial arrangements of genetic lineages. Swarm-founding wasps (Epiponini) as *Protonectarina sylveirae* (Saussure) are common representatives of Neotropical fauna. This species is widespread in Atlantic forest and presents a peculiar distribution within Epiponini because of its absence in Amazon region. Many studies point to the existence of recurrent phylogeographical discontinuities to different groups of organisms in Atlantic forest, but the explanations about events causing this pattern remain inconclusive. This work aimed to investigate the phylogeographic pattern of *P. sylveirae* with special interest in presence of genetic structure, morphological differences among populations, and historical event(s) which could explain the observed phylogeographic pattern. Specimens of *P. sylveirae* were actively collected in 13 areas throughout its distribution for DNA extraction and amplification of mitochondrial genes 12S, 16S and COI. Analysis of genetic diversity, historical demography, divergence time, and population structure were performed. Twenty-two haplotypes were identified, a strong genetic structuration was found by the analysis of molecular variance (ANOVA) and the haplotype network revealed three haplotype groups, also corroborated by AMOVA. Analysis of historical demography showed that populations of *P. sylveirae* had remained constant throughout its evolutionary history. Differences were also found in morphology, corroborating partially those found by molecular data. Divergence time suggests earlier orogenic events occurred during the Tertiary as an important cause driven population diversification of *P. sylveirae*, and the role of aridization of forested areas in modeling the current distribution of populations.

Keywords: Phylogeography. Epiponini. Neotropical region. mtDNA. Quaternary.

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APRESENTAÇÃO

Como disciplina que investiga os princípios e processos que determinam a distribuição geográfica de linhagens genealógicas, a filogeografia lida com as relações filogenéticas e os componentes históricos responsáveis pela distribuição de linhagens, criando uma ponte entre processos micro e macro-evolutivos. *Protonectarina sylveirae* (Saussure) é uma espécie com ampla distribuição (leste e centro-oeste do Brasil, alcançando também Argentina e Paraguai) pertencente à tribo Epiponini, uma tribo de vespas eussociais restritas e bastante comuns na região Neotropical. Uma vez que barreiras biogeográficas podem dificultar ou interromper o fluxo gênico entre as populações, pretende-se investigar a divergência entre as diferentes linhagens de *P. sylveirae* e a influência dos eventos geológicos que levaram a esta. As análises filogeográficas foram realizadas a partir de dados moleculares (3 genes mitocondriais e 1 nuclear) e morfológicos (8 medidas da morfologia externa do adulto). Os espécimes para as análises foram coletados ativamente com o auxílio de rede entomológica diretamente em flores ou nos ninhos, em áreas representativas da distribuição da espécie, escolhidas com base na literatura. Utilizou-se também amostras de coleção de museus. A partir dos dados moleculares, a relação entre os haplótipos encontrados foi observada por meio da construção de haplótipos. Foram realizadas análises para a inferência de eventos demográficos e uma análise de datação com o intuito de se estimar o tempo de divergência entre as linhagens. Os dados morfométricos foram analisados com o intuito de se averiguar a presença de divergência na morfologia de populações em localidades distintas. O objetivo do estudo foi realizar uma análise filogeográfica onde pretendia-se averiguar a ocorrência de estruturação genética associada à geografia e a presença de barreiras ao fluxo gênico. A presença de divergência na morfologia, associada à divergência genética e geográfica também foi averiguada. Objetivou-se ainda investigar os possíveis eventos que atuaram como barreiras, de acordo com o tempo de divergência encontrado.

1 PREFACE

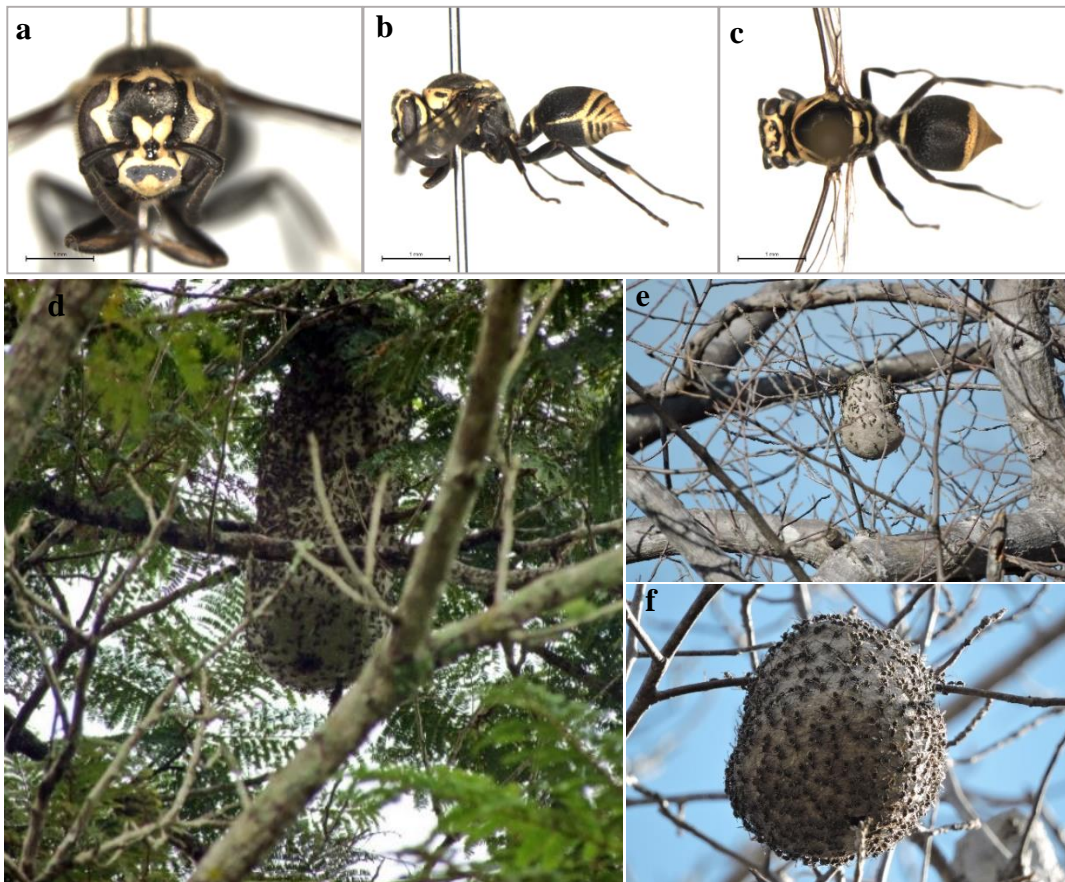
The comprehension of the evolutionary process that generates and keeps the biological diversity of tropical fauna has been challenging biologists for centuries and should underpin conservation strategies (MORITZ *et al.*, 2000). Current distribution results from the interaction between environmental requirements of species and geographical variation of environmental features (ESPÍNDOLA *et al.*, 2012). The origin of Neotropical biota cannot be explained by a single model of vicariance or recent climate changes (COSTA, 2003). Investigating the spatial patterns integrating to the molecular diversity, phenotypic variation, reproductive isolation and history of areas can provide information about speciation, clarify the historical biogeography and allow hypothesis regarding the diversification mechanisms. Thereafter, integrating such aspects can contribute significantly to the development of strategies for conservation of ecosystems (MORITZ *et al.*, 2000; KNOWLES, 2009).

These are the roles of phylogeography (AVISE *et al.*, 1987), which the goal is to characterize the phylogenetic deployment of genealogical lineages across the geographical landscape (ARBOGASTY & KENAGY, 2001). As the name suggests, it deals not only with phylogenetic relationship among taxa of interest but also with historical components responsible for the distributions. Moreover, it requires the use of a range of disciplines to interpret the data such as cladistics, population genetics, ethology, demography, paleontology, geology and historical geography (AVISE, 2000).

Neotropical social wasps (Polistinae) are highly diverse in terms of social organization, being mostly predators of other insects and floral visitors (NOLL, 2013). The group comprises more than 900 species, and the higher diversity is found in tropical and subtropical regions of the planet. Polistinae is divided in four tribes: Polistini, Mischocyttarini, Ropalidiini e Epiponini (NOLL, 2013). In Brazil, around 300 species of 21 genera are found (CARPENTER, 2003). *Protonectarina* Ducke is a monotypic genus of Epiponini, tribe restricted to Neotropics, including 19 genera (CARPENTER, 2003). Wasps of this tribe present as characteristics varied nest architecture, swarming foundation, polygyny (many functional queens) and complex caste differentiation (NOLL & WENZEL, 2008). *Protonectarina sylveirae* (Saussure) occurs throughout central region of Brazil (Minas Gerais, Mato Grosso do Sul e Goiás) and eastern of Brazil (Ceará, Bahia, Espírito Santo, Rio de Janeiro, São Paulo, Paraná, Santa Catarina e Rio Grande do Sul),

eastern end of Argentina and eastern Paraguai. Individuals of this species can be recognized for the large clypeus, eyes with many bristles and for the tergum I not petiolate in dorsal view (RICHARDS, 1978; CARPENTER & MARQUES, 2001) (fig.1, a-c). The species is remarkably for aggressiveness and for making very large and perennial nests, which are arboreal and usually suspended on a high branch, with the entrance in the ventral face of nest (WENZEL, 1998; SHIMA *et al.*, 2003) (figure 1). The nests may harbor a large population and a large amounts of nectar can be stored (RICHARDS, 1978; WENZEL, 1998; SHIMA *et al.*, 2003) (fig.1, d-f).

FIGURE 1 – DETAILS OF WORKER AND NESTS OF *Protonectarina sylveirae*.



a, front view of the head; **b**, lateral and **c**, dorsal; **d**, nest from Bonito – MS, **e**, nest from Mossoró – RN and **f**, detail of nest shown in **e**. Prepared by the author.

The Neotropical region covers elements of most of South America, including northern Argentina, and extends to the Caribbean, Central America and tropical areas of Mexico (AMORIM, 2012). During the last century, various biogeographical analyses were performed for South

America and many authors (CABRERA & YEPES, 1940; FITTKAU, 1969; RINGUELET, 1975; AB'SABER, 1977; RIVAS-MARTÍNEZ & NAVARRO, 1994; MORRONE, 2006) suggested different proposals of regionalization based on the fauna and flora of each region. Biogeographical regionalization are fundamental elements used by researchers who need to describe the distribution of a given taxon to, establish biotic comparisons between different areas or to refer to these areas for conservation. Therefore, it constitutes reference systems that are used by various disciplines (MORRONE, 2010).

Although endemic of the Neotropical fauna, studies of the history of the geographical distribution of Epiponini are scarce, however, it would be very useful to understand the history of this region. Because of the interesting eusocial biology of the neotropical wasps (Epiponini), features of its behavior has been explored exhaustively. However, other important aspects for understanding the evolution of the group remain underused. Phylogeography is an interesting field once interpretation of obtained data depends on an integrated approach with other subjects of biology. In addition to, know the genetic composition of species are important to direct studies on conservation.

Given the wide distribution in Atlantic Forest and the presence of possible barriers to genetic flow, this research aimed to characterize genetically populations of *Protonectarina sylveirae* throughout its distribution area to know how it is organized spatially. Differences on morphology were also investigated. The results found were evaluated and interpreted in a context of neotropical biogeography. As mentioned by ARBOGAST and KENAGY (2001), phylogeography is useful in elucidating contemporary patterns of evolutionary subdivision within species and species complexes and in providing novel insights into the relationship between earth history and biotic diversification.

2 INTRODUCTION

Ecological and historical processes of fauna diversification have been widely studied under the light of phylogeography, a relatively new discipline that deals with the spatial arrangements of genetic lineages, especially within and among closely related species (AVISE, 2009). The relationship between gene genealogies and geography can be used to estimate historical processes that can be responsible for contemporary geographic distributions of individuals and species (DANTAS *et al.*, 2011). The spatial structure of Neotropical biodiversity has long been studied by evolutionary biologists, particularly those interested in understanding the processes that could explain the origin of this diversification (SIGRIST & CARVALHO, 2009). This region includes the Central and South Americas, is known for its remarkably biodiversity (MORRONE, 2013) and it is perhaps the richest terrestrial biogeographical region in terms of species (CONDAMINE *et al.*, 2012).

Swarm-founding wasps - Epiponini – are common representatives of Neotropical fauna, with 19 genera and around 234 species described (NOLL, 2013). *Protonectarina* Ducke is a monotypic genus of this tribe and, *Protonectarina sylveirae* (Saussure) presents a broad distribution from Brazil to Argentina (Tanaka Jr. *et al.*, 2010), specifically along the central (Minas Gerais, Mato Grosso do Sul e Goiás) and eastern regions of Brazil (Ceará, Bahia, Espírito Santo, Rio de Janeiro, São Paulo, Paraná, Santa Catarina e Rio Grande do Sul), the eastern end of Argentina and eastern Paraguay. These wasps can be identified by broad clypeus, eyes with many setae and the tergum I not petiolate in dorsal view (RICHARDS, 1978; CARPENTER & MARQUES, 2001). They build arboreal nests, usually suspended from a branch and its colonies are large and store large amounts of nectar (RICHARDS, 1978; WENZEL, 1998; SHIMA *et al.*, 2003). A peculiar fact about the distribution of *P. sylveirae* is that within the genera of Epiponini this is the one that is not present in the Amazon region, occurring mainly in the Atlantic Forest. Most of the species of the tribe occurs in or is restricted to the Amazon (RICHARDS, 1978).

The Brazilian Atlantic Forest includes all forest physiognomies from north-eastern Brazil to its south, including coastal Atlantic rain forests, semi-deciduous forests, subtropical *Araucaria* (Jussieu) forests and *brejo* forests (SILVA *et al.*, 2012). For this area, many researches point to the existence of recurrent phylogeographical discontinuities to different groups of organisms for example: bees (BATALHA-FILHO *et al.*, 2010), amphibians (CARNAVAL *et al.*, 2009; THOMÉ

et al., 2010), reptiles (PELLEGRINO *et al.*, 2005; GRAZZIOTIN *et al.*, 2006), birds (CABANNE *et al.*, 2007; 2008), bats (MARTINS *et al.*, 2009) and plants (RIBEIRO *et al.*, 2011). Moreover, historical connections between Atlantic Forest and Amazonian rainforest have been inferred by some authors (COSTA, 2003; BATALHA-FILHO *et al.*, 2013).

The most well-known hypothesis about the origin of diversity in Brazilian Atlantic is based on the classical Pleistocene Refugia Model (HAFFER, 1969; VANZOLINI & WILLIAMS, 1981), which was postulated to explain the diversity in Amazon forest. In the last decade, interest in clarifying the origin of its biodiversity has increased (Silva *et al.*, 2012). The refugia hypothesis has been fully proposed and tested (CARNAVAL & MORITZ, 2008; CARNAVAL *et al.*, 2009), and other recent studies on the history of the Neotropical biota in general, searching for both temporal and spatial biogeographic patterns, also propose the uplift of the Andes, rise of land bridges between North and South Americas marine incursions, and Pleistocene climate changes (RULL, 2008; HOORN & WESSELING, 2010; HOORN *et al.*, 2010; RULL, 2011 and SILVA & NOLL, 2015) as events of diversification for Neotropical biota. All hypotheses are based in some provisional reductions gene flow among populations, which promoted divergence in allopatry, when the populations became different because they were somehow geographically isolated (DANTAS *et al.*, 2011).

Given the widespread (East and central region of Brazil) and particular distribution of *P. sylveirae* among Epiponini wasps (absent in Amazon region), the goal of the present study was to elucidate the phylogeographic pattern and demographic history of this species. We address some questions: Does populations of *P. sylveirae* presents genetical structuration? If yes, is this structure related to geography? Does this structuration also lead to the evolution of distinct morphologic lineages? Is there some historical event(s) in Neotropical region, especially in Atlantic Forest region, which explains the observed phylogeographic pattern?

3 MATERIAL AND METHODS

3.1 Sampling

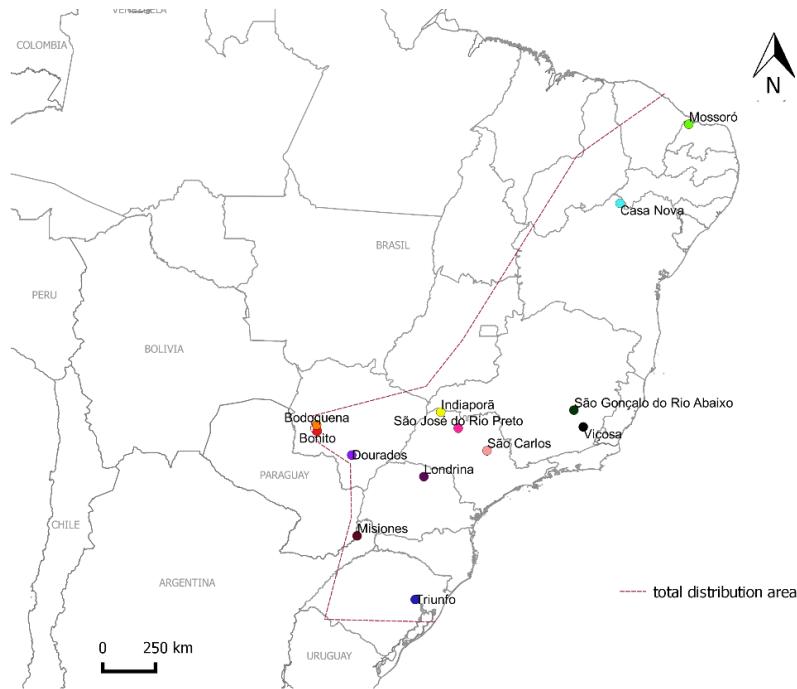
Specimens were actively collected in field with entomological nets in the vegetation or directly from nests and preserved in alcohol 100%. Areas were chosen according to the literature and at least ten individuals per area were sampled. Only workers were collected in order to avoid bias in morphometric analysis due to the dimorphism among castes (TANAKA JR. *et al.*, 2010). One sample was obtained from a museum (table 1, fig. 2). Although there were occurrence records for the cities of Leopoldo de Bulhões and Corumbá de Goiás, both in the state of Goiás (Brazil), nor nests of *Protonectarina sylveirae* or wasps flying were found in both municipalities. Another municipality near (Silvânia) was also visited, but again there were no wasps of this species. The records were obtained from material deposited in museum and dating from the ending of 30's. The large-scale cultivation of soybeans and corn in this region from the 60's may have led to a drastic decrease of the populations of this species in the region. Probably this species is very sensitive to environmental changes such as the removal of vegetation, as well as the use of pesticides.

TABLE 1 - LOCALITY, NUMBER OF INDIVIDUALS AND GEOGRAPHIC COORDINATES FOR SAMPLES OF *Protonectarina sylveirae*.

Areas	abreviation	n° of individuals	Latitude	Longitude
São José do Rio Preto, SP, BR	SJRP	10	-20.820	-49.378
Indiaporã, SP, BR	IND	10	-19.980	-50.289
São Carlos, SP, BR	SACA	10	-21.985	-47.881
São Gonçalo do Rio Abaixo, SP, BR	SGRA	10	-19.883	-43.367
Viçosa, MG, BR	VIC	8	-20.753	-42.881
Dourados, MS, BR	DOU	10	-22.211	-54.915
Bonito, MS, BR	BON	10	-20.967	-56.700
Bodoquena, MS, BR	BOD	10	-20.650	-56.733
Casa Nova, BA, BR*	CANO	10	-9.161	-40.970
Londrina, PR, BR	LON	10	-23.330	-51.165
Triunfo, RS, BR	TRI	10	-29.700	-51.616
Mossoró, RN, BR	MOS	10	-5.066	-37.400
Misiones, Argentina**	MIS	10	-26.400	-54.633
Total	13	128		

Samples from *American Museum of Natural History (AMNH, New York, USA - Dr. J.M.Carpenter) and **Faculdade de Filosofia Ciências e Letras (USP, Ribeirão Preto, Brazil - Dr. Sidnei Mateus). Coordinates are in decimal degrees.

FIGURE 2 – MAP OF BRAZIL AND CENTRAL PORTION OF SOUTH AMERICA.



Dotted line delineates the area of occurrence of *Protonectarina sylveirae*; colored circles, localities of samples. Prepared by the author.

3.2 DNA extraction, amplification and sequencing

DNA was extracted from legs, antennae and mesosomal muscles of wasps using the DNeasy Blood & Tissue (Qiagen) and Illustra tissue & cells genomicPrep mini spin kit (GE). The extracted DNA was used to amplify part of the mitochondrial genes cytochrome oxidase subunit I (COI) and 16S and 12S ribosomal RNA, and nuclear gene Elongation factor alpha 1 (EF 1- α). PCR was performed using the “Pure Taq Ready-To-Go PCR beads” (GE) kit. Primers used to amplify sequences of COI and 16S were developed by SCHULMEISTER *et al.* (2002) while for the 12S gene a primer were constructed (table 2, appendix A).

PCR products were purified employing “GFX PCR DNA and Gel purification” (GE) kit. Sequencing was done in the “Centro de Recursos Biológicos e Biologia Genômica” (CREBIO/UNESP Jaboticabal, SP) using the automatic sequencer ABI (Applied Biosystems, Foster City, CA) to be sequenced. Sequences were aligned in Mafft server (<http://mafft.cbrc.jp/alignment/server/>).

TABLE 2 - SEQUENCE OF PRIMERS USED TO AMPLIFY FRAGMENTS OF GENES.

Gene	Direction	Name	Sequence (5' - 3')	length*
COI	F	JERRY	CAACATTTATTTTGATTTTTTGG	~ 850bp
	R	PAT	TCCAATGCACTAATCTGCCATATTA	
	F	LCO	GGTCAACAAATCATAAAGATATTGG	~ 740bp
12S	R	HCOoutout	GTAAATATATGRTGDGCTC	~ 400bp
	F	12SF	ACTWTGTTACGACTTATTYC	
16S	R	12SR	AAACTAGGATTAGATACCCTATTAT	~ 515bp
	F	16SA	CGCCTGTTTATCAAAAACAT	
EF1- α	R	16SB	CTCCGGTTTGAACCTAAGATCA	~ 1075
	F	HAF2for1	GGGYAAAGGWTCCCTTCAARTATGC	
	R	F2REV1	AATCAGCAGCACCTTTAGGTGG	

F, *forward*; R, *reverse*; bp, base pair; *approximate size of the amplicon. COI, cytochrome oxidase subunit I; 12S ribosomal RNA, 16S ribosomal RNA and EF1- α , Elongation factor alpha 1.

3.3 Genetic diversity

In order to quantify the variation in DNA sequences and characterize populations, the following parameters were estimated in DnaSP v. 5.101 (ROZAS *et al.*, 2010): number of polymorphic sites (**S**), nucleotide diversity (π), average number of nucleotide differences (**k**), total number of mutations (η), number of haplotypes (**h**) and the haplotype diversity (**Hd**). To investigate if the genetic divergence could be explained by isolation by distance the Mantel test (MANTEL, 1967) was applied in IBDWS (Isolation by Distance Web Service) 3.23 (JENSEN *et al.*, 2005), with (1000) replications.

3.4 Haplotype relationship and population structure

For the purpose of verify the relationship among the haplotypes a gene genealogy was reconstructed in the program Network 5 (fluxus-engineering) using the median-joining (BANDELT *et al.*, 1999) algorithm to describe graphically the relationship between the distribution of haplotypes and the geographic distribution of the populations of *P. sylveirae*.

An Analysis of Molecular Variance (AMOVA) was performed to test the presence of population structure. AMOVA is based on F-statistics and assumes the hypothesis that the genetic diversity within the two populations is not significantly different from that resulting from the

joining of two populations (EXCOFFIER, *et al.*, 1992; ANDERSON, 2001). Two approaches were used: considering sample sites, without any *a priori* population group and second, dividing populations in groups resulting from the groups in the haplotype nests. Analysis was done in Arlequin, version 3.5.1.3 (EXCOFFIER & LISCHER 2010).

3.5 Demographic events

Neutrality tests D de Tajima (TAJIMA, 1989a, b) and Fu's F_s (FU, 1997) were performed in Arlequin 3.5.1.3 (EXCOFFIER & LISCHER, 2010) to detect signs of population expansion or bottlenecks. A value of $D = 0$ implies neutrality, while a significant value of D can indicate population expansion, bottleneck or heterogeneity in mutation rates (TAJIMA, 1996). A high negative value of F_s indicates population expansion. The distribution of genetic differences between pairs of haplotypes (*Mismatch* distribution) was performed considering the premise of panmictic populations using the program DnaSP v. 5.101 (ROZAS *et al.*, 2010). In general, multimodal distributions are consistent with demographic stability or multiple expansion events, while unimodal distributions commonly indicate that the population underwent a recent population and spatial expansion (ROGERS & HARPENDING, 1992). The Mismatch distribution was performed for each population and using the haplotype sequences.

3.6 Divergence time

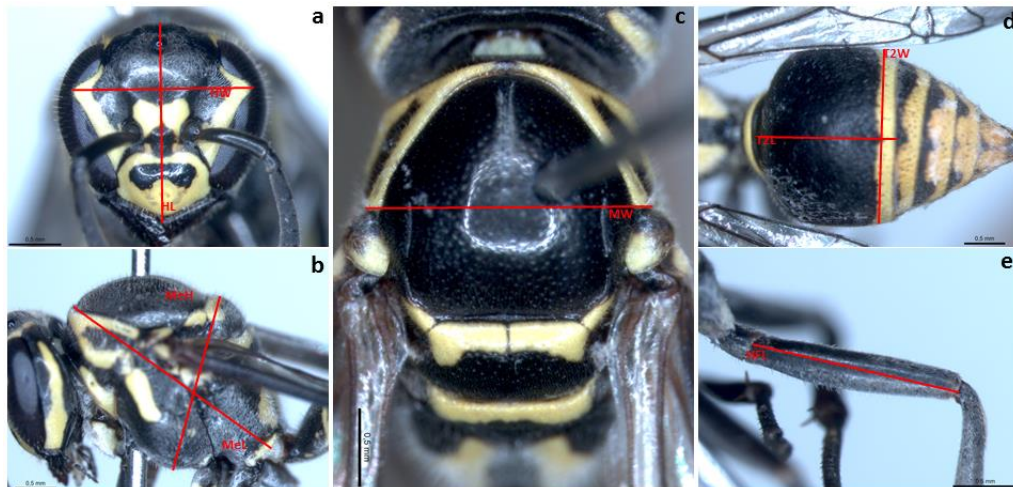
The time since the most recent common ancestor (TMRCA) was estimated under a Bayesian approach using BEAST 1.8.3. (DRUMMOND *et al.*, 2012). Analyses were conducted using sequences of COI gene for populations plus sequences of 3 other species of Epiponini (*Agelaia testacea*, *Synoeca surinama* and *Brachygastra augusti* as outgroups) under the GTR nucleotide substitution model, found as the best model for the data in MEGA 7.0.7. (KUMAR *et al.*, 2016). The age of fossil species *Agelaia electra* (20.43 to 13.65 Ma) (CARPENTER & GRIMALDI, 1997) was used for calibration, considering a mean of 17.04 Ma ± 0.5 . Analyses were conducted using default parameters for MCMC, setting 10 million generations with 10% of the initial runs were excluded. The resulting trees were combined using TreeAnnotator v1.8.0 and the consensus tree with the divergence times was visualized in FigTree v1.4.0 (RAMBAUT, 2009).

3.7 Body size morphometry

Because of the absence of recombination and high rate of evolution, mitochondrial markers are highly informative in evolutionary studies and well suited for intraspecific analysis of genetic divergence (AVISE, 2000). However, morphological characters are known to be frequently affected by natural (and sexual) selection and therefore might provide information on local selective pressures (HABEL *et al.*, 2013) and indicate a gene flow rupture history of the various populations due to a partial or total isolation between them.

Digital images of the body of 128 individuals of *P. sylveirae* from the 13 populations sampled were taken in stereo microscope coupled with digital camera (Leica PDF295) before the DNA extraction. The follow adult body parts (fig. 3) were chosen based on work of Garcia *et al.* (2008): Head length (HL), head width (HW), mesoscutum width (MW); mesosoma length (MeL), mesossoma height (MeH), hind femur length (HFL); 2° metasomal terga length (T2L) and 2° metasomal terga width (T2W).

FIGURE 3 – BODY SIZE MEASURES FOR MORPHOLOGICAL ANALYSIS OF POPULATIONS OF *Protonectarina sylveirae*.



a, Head length (HL) and head width (HW); **b**, mesosoma length (MeL) and mesossoma height (MeH); **c**, mesoscutum width (MW); **d**, 2° metasomal terga length (T2L) and 2° metasomal terga width (T2W) and **e**, hind femur length (HFL). Pictures from an individual from population of Mossoró, RN. Prepared by the author.

ImageJ software (<http://imagej.nih.gov/ij/index.html>) was used to derive the measures in the images. Comparisons between populations within species were performed. To verify the degree of discrimination and identify which measures better explain the data a multivariate discriminant

analysis, with a posterior canonical discriminant analysis was done (ARAUJO *et al.*, 1998) The tests were performed with the help of program STATISTICA 7 (StatSoft, 2004).

4 RESULTS

4.1 Characterization and diversity of the sequences

The final alignment containing the concatenated data of 12S, 16S and COI result in a matrix with 112 sequences and 2.468 base pairs. For the nuclear gene Elongation factor 1- α , a fragment of 1324 base pairs was sequenced. However, this gene did not show any intraspecific polymorphism and only the mitochondrial data was used.

Number of variable sites (S) were 161 and the total number of mutations (η) 162. A total of 22 haplotypes (h) (table 3, appendix B) were identified with a diversity (Hd) of $0,894 \pm 0,016$. The difference in the number of nucleotides between sequences averaged 37,74 and the nucleotide diversity (π) was $0,015 \pm 0,00089$ across all populations. Haplotype 5 is the most widespread, being shared among the populations of IND, BOM and BOD. Populations of SGRA and VIC share haplotype 1 (table 3). The high haplotype diversity and low nucleotide diversity values indicate only small differences between haplotypes, that is, each population carries a different part of the total amount of diversity of species.

Mantel test showed no correlation for the logarithm of genetic distance and the geographical distance ($r = 0.4320$; $p < 0.9816$), indicating that the genetic divergence among populations cannot be attributed to isolation by distance (appendix C).

Table 3. Genetic diversity and neutrality tests for each population of *Protonectarina sylveirae*. Significant values are presented in bold. N, size of sample; Hd, haplotype diversity; π , nucleotide diversity.

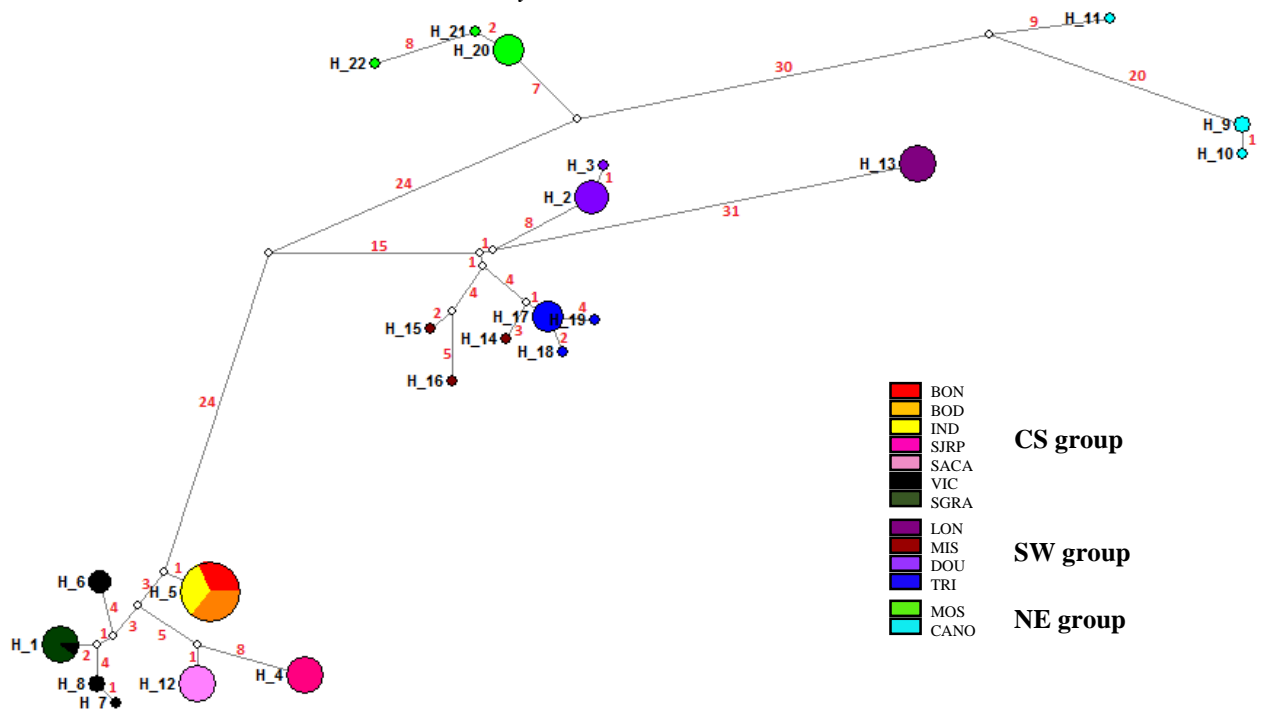
Population	N	Haplotypes (frequency)	Neutrality tests				Groups	Hd	π
			Tajima's D	p-value	Fu's FS	p-value			
SGRA	9	Hap_1 (10)	0.00000	1.00	0.00000	n.a.	CS group	1	0,07516±0,01237
VIC	8	Hap_1 (4), Hap_6 (1), Hap_7 (1), Hap_8 (2)	1.438336	0.93	2.42794	0.878			
SJRP	10	Hap_4 (10)	0.00000	1.00	0.00000	n.a.			
SACA	10	Hap_12 (10)	0.00000	1.00	0.00000	n.a.			
IND	9	Hap_5 (9)	0.00000	1.00	0.00000	n.a.			
BON	9	Hap_5 (9)	0.00000	1.00	0.00000	n.a.			
BOD	10	Hap_5 (9)	0.00000	1.00	0.00000	n.a.			
DOU	10	Hap_2 (9), Hap_3 (1)	-1.11173	0.192	-0.33931	0.148	SW group	1	0,11145±0,02933
LON	10	Hap_13 (10)	0.00000	1.00	0.00000	n.a.			
MIS	3	Hap_14 (1), Hap_15 (1), Hap_16 (1)	0.00000	0.681	1.20789	0.477			
TRI	10	Hap_17 (8), Hap_18 (1), Hap_19 (1)	-1.56222	0.03	0.83799	0.649			
MOS	10	Hap_20 (8), Hap_21 (1), Hap_22 (1)	-1.53448	0.061	2.35538	0.903	NE	1	0,24350±0,04326
CANO	4	Hap_9 (2), Hap_10 (1), Hap_11 (1)	-0.86148	0.047	3.31896	0.894	group		

BOD, Bodoquena-MS; BOM, Bonito-MS; CANO, Casa Nova-BA; DOU, Dourados-MS; IND, Indiaporã-SP; LON, Londrina-PR; MIS, Misiones-Argentina; MOS, Mossoró-RN; SACA, São Carlos-SP; SGRA, São Gonçalo do Rio Abaixo-MG; SJRP, São José do Rio Preto-SP; TRI, Triunfo-RS and VIC, Viçosa-MG.

4.2 Haplotype relationship and population structure

Figure 4 shows the phylogenetic relationships among haplotypes of *P. sylveirae* based on the haplotype network. It is evident in the network a particular haplotype composition for each population, with only haplotype 1 been shared between populations of SGRA and VIC, and haplotype 5 shared by populations of BON, BOD and IND. Nevertheless, two distinct groups are evident: a south-west group (SW) with population from DOU, LON, MIS and TRI, and a central southeast group (CS) grouping populations from SGRA, VIC, BOM, BOD, IND, SJRP and SACA. Because of their localities, populations of CANO and MOS are considered as the northeast group (NE) despite being separated by a large number of mutations, in comparison with populations from two other groups. Based on Network result, further analyses were performed considering also these tree groups of populations.

FIGURE 4 – HAPLOTYPE NETWORK INFERRED FOR 22 HAPLOTYPES (H) OF THE 12S, 16S AND COI GENE OF *Protonectarina sylveirae*.



Haplotype nest estimated by the median-joining algorithm. Circles are proportional to the frequency of haplotypes and colors represent each population sampled. Numbers in red indicate mutational steps between haplotypes. Empty circles represent hypothetical haplotypes (lost or unsampled). Some branches were moved for better visualization. BOD, Bodoquena; BON, Bonito; CANO, Casa Nova; DOU, Dourados; IND, Indiarorã; LON, Londrina; MIS, Misiones; MOS, Mossoró; SACA, São Carlos; SGRA, São Gonçalo do Rio Abaixo; SJRP, São José do Rio Preto; TRI, Triunfo and VIC, Viçosa. CS, Central southeast; SW, South-west; NE, Northeast. Prepared by the author.

Analysis of Molecular Variance (AMOVA) results in a high value of F_{ST} (0,966). The source of variation was 96,62% among populations and only 3,38% within them. When the analysis was repeated dividing population in the 3 groups (NE, SW and CE), F statistics value was even higher (0,9747). Percentage of variation was 65% among groups, 32,43% among populations within groups and just 2,52% within populations (table 4). All the above results suggest a strong genetic structure between groups and support a model of low gene flow.

TABLE 4 – F STATISTICS' VALUES RESULTING FROM AMOVA FOR GLOBAL AND PRE-DEFINED GROUPS.

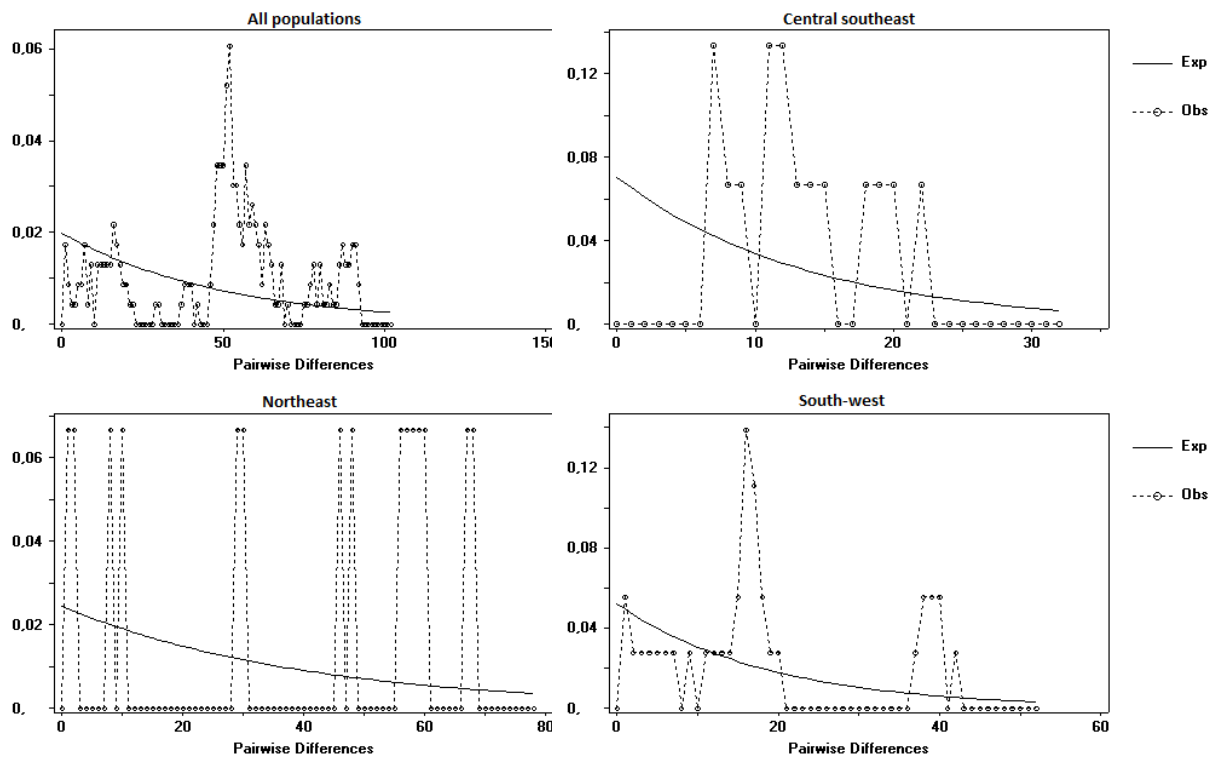
	F statistics					
	FST	p-value	FSC	p-value	FCT	p-value
Global	0,9662	0,0000	---	0,0000	---	0,0000
Groups	0,9747	0,0000	0,9277	0,0000	0,6504	0,0000

$P < 0,05$ were considered significant.

4.3 Demographic events

Values of F_u 's F_s were not significant for populations. For Tajima's D , populations of CANO, TRI and MOS showed a significant negative value, indicating an excess of rare alleles within the population and may suggest recent population expansion or purifying selection (Ramos-Onsins & Rozas 2002; Hartl & Clark 2010). Neutrality tests performed for each group was not significant (table 3). The *Mismatch* distribution results in a multimodal distribution. Analysis was repeated per haplotype groups (the same used in AMOVA) and the same pattern was found for all groups (fig. 5).

FIGURE 5 - GRAPHICS OF *MISMATCH* DISTRIBUTION FOR ALL POPULATIONS OF *Protonectarina sylveirae* TOGETHER AND FOR HAPLOTYPE GROUPS.



Prepared by the author.

4.4 Divergence time

The divergence dating analysis revealed a middle Miocene origin for *Protonectarina*. The oldest divergence occurred between lineages of northeast (NE) and the remaining (at 11 Ma), followed by the separation between lineages from south-west (SW) and central southeast (CS) (around 10 Ma). At 9 Ma the lineage of LON became separated from the others of SW group and around the same time SGRA+VIC diverge from the others of CS group. Divergence of NE lineages occurred 1 Ma later and, a little later, lineages of SACA and SJRP diverge from the remaining middle-west lineages within CS group (fig. 6, appendix D).

TABLE 5 - CLASSIFICATION MATRIX SHOWING FOR EACH POPULATION THE NUMBER OF CASES CLASSIFIED CORRECTLY AND INCORRECTLY ACCORDING THE DISCRIMINANT MODEL.

	%correct	1	2	3	4	5	6	7	8	9	10	11	12	13
1 SGRA	80	8	0	0	1	0	0	1	0	0	0	0	0	0
2 DOU	100	0	10	9	0	0	0	0	0	0	0	0	0	0
3 SJRP	90	0	0	0	0	0	0	0	0	1	0	0	0	0
4 IND	70	0	0	0	7	0	0	0	0	0	2	1	0	0
5 VIC	37,5	0	0	0	1	3	0	2	0	1	0	0	0	0
6 BON	60	0	0	0	0	0	6	1	0	0	0	0	0	2
7 BOD	70	0	0	0	0	0	3	7	0	0	0	0	0	0
8 CANO	100	0	0	0	0	0	0	0	10	0	0	0	0	0
9 SACA	60	0	0	0	0	0	1	0	0	6	2	0	0	1
10 LON	30	2	0	0	0	0	0	0	0	1	3	3	0	0
11 MIS	50	0	0	1	0	0	1	0	0	0	3	5	0	0
12 TRI	70	0	0	1	0	0	0	0	0	0	2	0	7	0
13 MOS	50	1	0	1	0	1	1	1	0	1	0	0	0	5
TOTAL	67,187	11	10	14	9	4	12	12	10	10	12	9	7	8

Rows: observed classifications; columns: predicted classifications.

Contribution of each variable (measure) in functions generated by the value of Wilks' λ were verified to see which of them were important to discrimination among groups (populations) and only the measures MeL and MeH were not significant (table 6).

TABLE 6 – DISCRIMINANT FUNCTION ANALYSIS SUMMARY.

	MW	T2W	HFL	LH	WH	T2L	MeH	MeL*
Wilks' λ	0,0500	0,0053	0,0056	0,0066	0,0064	0,0045	0,0040	0,0030
Partial λ	0,0673	0,6263	0,5958	0,5060	0,5238	0,7342	0,8330	0,9026
F	123,46	5,3202	6,0471	8,7023	8,1044	3,2269	1,7864	0,9528
p-value	0,0000	0,0000	0,0000	0,0000	0,0000	0,0005	0,0594	0,4982

N° of variables in model: 7 (* indicates a variable currently not in the model); Grouping: localities (13 localities) Wilks' λ : ,00337 approx., F (84,663) = 12,106; p<0,0000 is considered significant. HL, head length; HW, head width; MW, mesoscutum width; MeL, mesosoma; MeH, mesosoma height; HFL, hind femur length; T2L, 2° metasomal terga length and T2W, 2° metasomal terga width.

Analysis of standardized coefficients (table 7), identifies MW as the most relevant in delimitation of the groups, being the measure with the higher contribution in the share of discrimination (about 80%) represented by two functions discrimination between

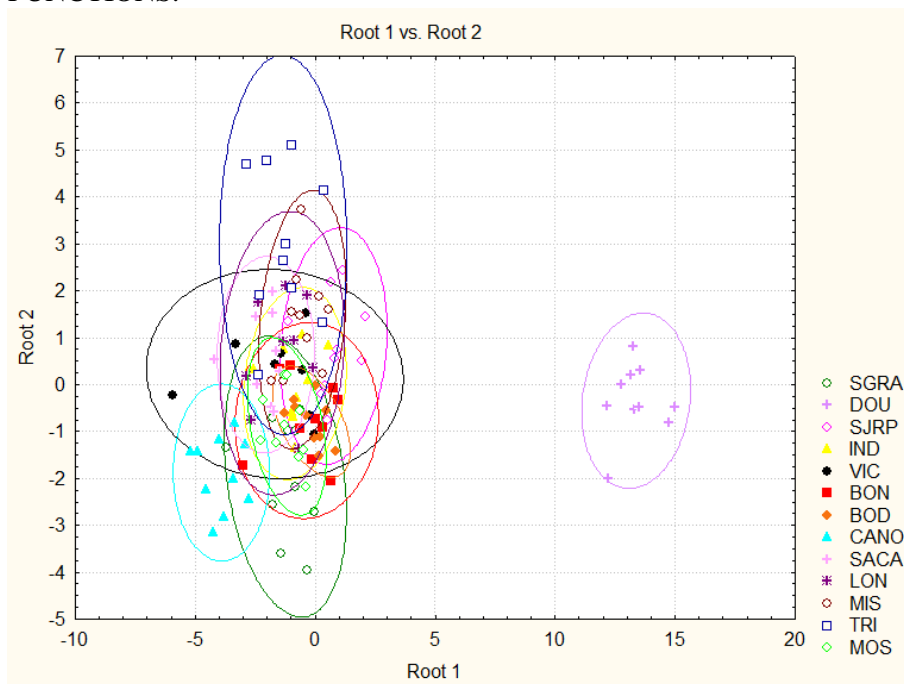
groups (Root 1 and Root 2). The ordination plots (fig. 7) show an indication of geographical variation, explained mainly by the shape. A remarkably difference between populations of DOU as well as population of CANO, though not so conspicuously, can be observed. For the remaining population the plots show no existence of discrete clusters of individuals, with some degree of overlap among them. However, it is possible to observe that populations of SW group (LON, MIS and TRI) are distributed from 0 to positive values of root 2 axis, while majority of populations of CS and NE groups are below 0 (except VIC, SJRP and SACA) (fig. 8), what means that morphologically there is some distinction between SW group and CS + NE groups.

TABLE 7 – STANDARDIZED COEFFICIENTS FOR THE CANONICAL VARIABLES.

	Root 1	Root 1	Root 1	Root 1	Root 1	Root 1	Root 1
MW	-1,1656	0,0884	-0,1728	0,0884	0,0289	-0,0198	0,0491
LT2	0,3227	-0,5066	0,3622	0,5529	-0,5484	0,1271	0,5188
HFL	-0,3360	0,6828	0,4123	-1,0566	-0,2312	-0,1554	0,4351
LH	0,0499	-1,5386	-0,3723	-0,5556	0,7282	-0,6956	0,3374
WH	0,2538	1,3297	0,4361	1,1636	-0,1126	-0,3059	-0,4512
T2L	0,1760	-0,1121	0,2878	0,3225	0,7893	0,6920	0,0973
MeH	0,1855	-0,3224	0,3261	-0,1857	-0,4052	0,2809	-1,0152
Eigenval	18,5192	1,9152	1,1698	0,4240	0,3372	0,2184	0,0352
Cum. Prop.	0,8187	0,9034	0,9551	0,9738	0,9887	0,9984	1,0000

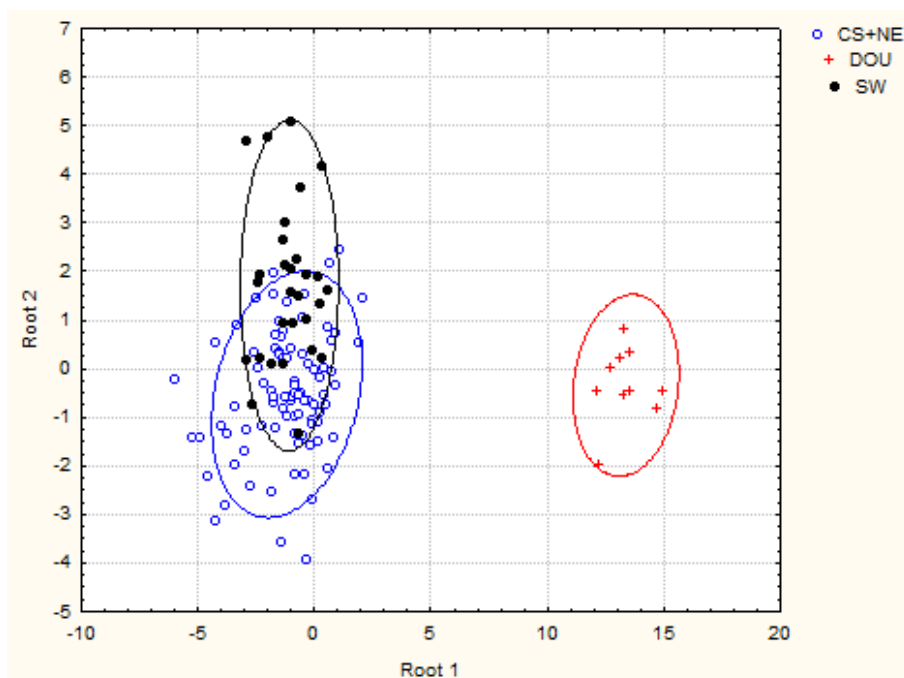
Numbers in bold show the contribution of the two functions that best explained the discrimination found among populations. HL, head length; HW, head width; MW, mesoscutum width; MeL, mesosoma; MeH, mesosoma height; HFL, hind femur length; T2L, 2° metasomal terga length and T2W, 2° metasomal terga width.

FIGURE 7 – DIVERGENCE PATTERN OF GROUPS ACCORDING DISCRIMINANT FUNCTIONS.



Legend: Shapes represents populations. SGRA, São Gonçalo do Rio Abaixo; DOU, Dourados; SJRP, São José do Rio Preto; IND, Indiaporã; VIC, Viçosa; BON, Bonito; BOD, Bodoquena; CANO, Casa Nova; SACA, São Carlos; LON, Londrina; MIS, Misiones; TRI, Triunfo and MOS, Mossoró.

FIGURE 8 – DIVERGENCE PATTERN OF GROUPS ACCORDING DISCRIMINANT FUNCTIONS SHOWING THE UPPER POSITION OF POPULATIONS OF SW.



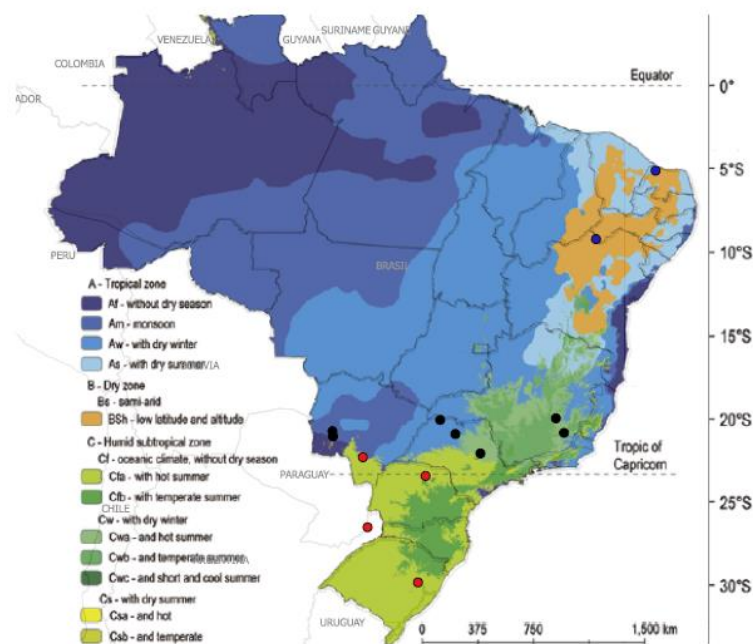
Legend: SW, Southwest group; CS+NE, Central Southeast + Northeast groups; DOU, Dourados.

5 DISCUSSION

5.1 Distribution, demography and genetic and spatial structure

The results obtained for *Protonectarina sylveirae* in the present study clarify the evolutionary history and the pattern of genetic variability for the species. A first interesting information regarding the spatial localization and restriction of haplogroups is that it seems to be related with climate. Distribution of haplogroups are strongly congruent with Köppen's climate classification, with SW group under the humid subtropical zone without dry season, NE group under the semi-arid zone with low latitude and altitude and CS group under tropical zone with dry winter and humid subtropical zone with dry winter and hot summer (ALVARES *et al.*, 2013) (fig. 9).

FIGURE 9 – CLIMATE CLASSIFICATION OF BRAZIL ACCORDING KÖPPEN (1936) CRITERIA.



Populations from haplogroups under climate zones. Northeast (blue dots), Central+Southeast (black dots) and Southwest (red dots). Adapted from (ALVARES *et al.*, 2013)

Across the range of its distribution, populations are genetically highly structured, as confirmed by AMOVA and presumably reflecting long lasting isolation in the past. Populations within the same geographical region are genetically similar, as populations of BON and BOD, or presents a low degree of genetic divergence, suggesting restricted gene flow between geographically separated groups of populations. However, the

particular haplotype composition for majority of populations inside the same geographical region imply a condition of restricted gene flow not related with isolation by distance, but probably caused by the presence of a barrier. The division in three mainly previously defined groups were also confirmed by AMOVA.

The type of social organization in Vespids has generated profound effects in several aspects of the group (GARCIA, *et al.*, 2008). Non-swarm-founding species can generate new colonies in a shorter time and their dispersion could be easier; however, the colony success is lower given the low number of founding individuals. By contrast, a swarm-founding species, as *P. sylveirae* reduces queen mortality risk, and increases homeostatic control of the colony, but the dispersal process could be more restrictive once populations could be prone to local extinction since the swarm is more vulnerable to harsh climatic conditions for the migration (JEANNE, 1991; Bouwma *et al.*, 2003). Nests of *P. sylveirae* are rare and difficult to be located in the field. They usually build them in high trees and are present only in areas with forested vegetation. Even specimens from CANO and MOS, located on biome of Caatinga, were found in high trees. These biological requirements should have shaped the distribution of populations and contribute with the maintenance of low rates of gene flow. Areas with open vegetation and/or dry conditions probably were not a barrier for the expansion of populations but they were for the establishment of colonies. Sufficient gene flow between populations can slow down or prevent the process of geographic differentiation, and leave a signature of little population structure over large areas. This is commonly observed in flying insect species, specifically in those species that migrate or are good dispersers (JONG *et al.*, 2011). Nevertheless, this pattern was not observed for *P. sylveirae*.

The divergence among haplotypes and presence of genetic structuration suggests a deep divergence instead of ancestry based on coalescence, which means low rates of genetic flow, resulting in the high index of F_{ST} . Taking into account the predictions of the theory of coalescence, there is a direct relationship between haplotype frequency and the age of haplotypes where the most common haplotypes are the oldest (FERRERI *et al.*, 2011). Hence, the majority of new mutants are derivatives of common haplotypes, that is, rare variants are most recent mutations and most closely related to the common haplotypes than to the other rare variants (EXCOFFIER & LANGANEY, 1989). It was not possible to determine the haplotype ancestry of groups of haplotypes. Topology of network shows the presence of many hypothetical haplotypes, which can be attributed to

the loss of this haplotypes in a past time. Notwithstanding, it cannot be discarded a lack in sample, mainly in the area close to the southeast coast, which was not sampled.

Rapid cycles of extinction and colonization can also affect the genetic structure of populations, where frequent founding events during colonization may increase the value of F_{ST} (WRIGHT, 1940). But this value depends on the settlement mode, more specifically the source of the origin of migrants (SLATKIN, 1977). The combination of high haplotype diversity and low nucleotide diversity, as observed in our data, also can be a signature of a rapid demographic expansion from a small effective population size (AVISE, 2000). In eusocial species such as *P. sylveirae*, only a few individuals of colony are reproductive. After a swarm, colonies start with several queens and workers, and as the colony develops, some queens disappear or adopt a worker role and the number of queens reduces until one or a few queens remain at last (NOLL, 2013).

A negative value of Tajima's D , as found for populations of MOS and TRI suggests a population expansion but can also be obtained if the population experienced a bottleneck recently (TAJIMA, 1989a). Although Tajima's D is relatively sensitive to demographic expansion, the simulations performed by Ramos-Onsins & Rozas (2002) showed that it tends to have less power than F_s (Fu 1997) and R_2 (RAMOS-ONSINS & ROZAS 2002) in scenarios where time since expansion is high and/or the sample size and/or the growth rate are low (D-HORTA *et al.*, 2011). On the other hand, the multimodal Mismatch distribution suggests stability in populations (SLATKIN & HUDSON, 1991; ROGERS & HARPENDING, 1992). However, recently changes in size of population cannot be detected due to threshold effects, lapses of time or previous demographic events, which can mask the effects of recent events (JOHNSON *et al.*, 2007). Multimodal distribution also can be generated for populations that suffered recent population expansion but are subject to higher migration rates and/or suffered historical population reduction (CASTOE *et al.*, 2007; NUÑEZ *et al.*, 2011).

As found by *P. sylveirae*, a high H_d and a low π also were found by Carvalho (2014) for *Angiopolybia pallens* and *Synoeca surinama*, two species of paper wasps also belonging to Epiponini.

Comparing with *A. pallens*, a lower genetic diversity was found for *S. surinama* and the authors attributed it to the differences in biology between two species (e.g. vagility, body size and distinct colonization systems). Another similar result was the presence of many hypothetical haplotypes, which didn't allow the inference of ancestry among haplotypes. It is not possible verify structuration in a local degree for these species

once haplotypes were represented per putative refuges areas. However, besides the author argue in favor of differences in biology as the cause of disjunctions, a clearly structuration between the two areas of forest, Amazonian and Atlantic forest can be observed for both species, suggesting the influence of a historical component. Interestingly, for *S. surinama*, which also occurs in Brazilian savannah, haplotypes for this biome formed a group with Amazon, but haplotypes of populations of Atlantic forest remained closer. Anyway, *Angiopolybia* and *Synoeca* are genera whose diverge earlier than *Protonectarina* (WENZEL & CARPENTER, 1994) and also showed differences in many aspects as nest construction, number of individuals in colony and behavior.

Morphometric analysis indicates the presence of differences among populations, which were statistically significant. Furthermore, according to the results it could be seen that the measures chosen were adequate for discrimination of groups. From the graphic of discriminant analysis, it was possible to observe a difference in the distribution of plots between populations of SW group and the remained populations. This fact corroborates a division in at least the two groups of CS and SW and is in agreement with molecular data.

The divergence standards for molecular markers depends on the selection acting on DNA. It ranges from functional genes under strong stabilizing selection, decreasing the rate of evolution of DNA, to the non- functional regions which rapidly evolves according neutral evolution model. Likewise, the natural selection can force morphological evolution away from this expected correlation. Its possible outcomes ranging from cryptic lineages resulting from stabilizing selection to high rates of morphological divergence as a result of directional selection (RENAUD *et al.*, 2007).

5.2 Time, diversification and historical aspects

The dating analysis showed a time of divergence previous to the Pleistocene and therefore, not consistent with the classical Refugia Model. Time tree shows a sequence of divergence of lineages follow NE(SW+CS), with the first divergence occurring between NE/(SW+CS) and passing around 11 Ma. Since late Tertiary (11 to 5 Ma) a mesophytic forest corridor have crossed the Caatinga at certain periods (RIZZINI, 1963; ANDRADE-LIMA, 1964), which is congruent with the presence of populations of *P. sylveirae* in this region earlier. In addition, the called “Brejos de Altitude”, montane forests that presently exist within the semi-arid region, are relicts of an ancient and wider

forest cover and represent current forest refuges during the Plio-Pleistocene (BIGARELLA & ANDRADE-LIMA, 1982; BATALHA-FILHO, 2013). It should be clear that the present Caatinga vegetation is inhospitable to most rainforest species, and even more stressful to arboreal ones and (VIVO, 1997). However, Quaternary period is a time too young to explain the cause of divergence between areas of northeast, the isolation of forested areas probably helps to increase the genetic divergences between these populations.

Increasing in dryness in South America starting since Oligocene and Miocene allowing expansion of savanna-like vegetation, evidenced by paleo floral records from later Oligocene (SAFFORD, 1999). This period of high temperature and dry climate had marked biogeographic and evolutionary implications (SOUZA *et al.*, 2003). Especially for southeastern Brazil in particular, periods of forest contraction forced populations of plant and animal species adapted to a wet climate to become isolated in wet areas, mainly in valleys (SAFFORD, 1999; ZANELLA, 2012). This fact can be contributed for the genetic differentiation among lineages of *P. sylveirae* in these areas.

Ridges and mountain uplifts are well-known mechanisms of gene flow interruption and promote divergence among populations (TURCHETTO-ZOLET, *et al.*, 2012). During Pliocene and Miocene, southeastern Brazil was subjected to intense orographical activity resulting in Serra do Mar and Serra da Mantiqueira mountain uplifts (ALMEIDA, 1976; SUGUIO & MARTIN, 1976). The uplift of Serra do Mar possibly interrupted precipitation in southeastern Brazil by the early Pliocene at about 5.6 Ma and therefore altered the distribution of humid and dry habitats. This period coincides with the transition from tropical humid to semiarid or arid conditions described by some authors (SIMPSON 1979; VASCONCELOS *et al.* 1992). This orogenic process deeply changed the geomorphologic and climatic conditions of south and southeast areas of Brazil, and consequently fragmented Brazilian Atlantic Forest with drier areas (GRAZZIOTINI *et al.* 2006; DANTAS *et al.*, 2011). After geologic disruption, populations may have experienced periods of isolation by ridges and valleys typical of present day relief (SOUZA *et al.*, 2003). Despite the spatial congruence of some populations of *P. sylveirae* in Serra da Mantiqueira, a very ancient origin is attributed to this geomorphological formation and it cannot be invoked as the cause of the divergence between groups.

Population of DOU belongs to the SW haplogroup. These populations share the Parana watershed and a common climate region. However, this population appears quite

different morphologically from the others, including those of the same group. A possible cause is the Paraná river, which is between DOU and the remaining SW populations, hindering the gene flow and reinforcing the difference among them. Another interesting result was that, despite the spatial proximity, population of DOU do not belong to the same haplogroup of populations of BON and BOD. However, BON and BOD are in a different watershed (basin of Paraguay). Moreover, while DOU is in the plateau zone of state of Mato Grosso do Sul, BON and BOD are in Serra da Bodoquena, two different geological formations.

Previous studies with other genera of Epiponini found a more recently divergence time. A phylogeographic analysis of *Angiopolybia pallens* and *Synoeca surinama* (CARVALHO, 2014) found an early origin for the species, around 3 and 2 Ma respectively. Menezes *et al.* (2015) found a middle/late Miocene origin for *Synoeca*, with subsequent diversification of extant species occurring in the Plio-Pleistocene. The calibration used was different for both works and also differ in this study. The inference of lineage divergence time from molecular data has been criticized, mainly because dating is prone to many types of error (particularly for those taxa lacking an available fossil record for calibration) (TURCHETTO-ZOLET *et al.*, 2013), one of which is related to the highly influence of calibration scheme (SAUQUET *et al.* 2012). Hence, the patterns reported here must be evaluated carefully.

Single locus estimation of population divergence should always be treated with caution as it can be overestimated due to polymorphism in the ancestral population and could present a wide variance due to the stochastic nature of the lineage sorting process (JENNINGS & EDWARDS, 2005; GRAZZIOTIN *et al.*, 2006). However, deep divergence times as estimated here are usually not as affected by ancestral polymorphism as more recent events (ARBOGAST *et al.* 2002). In spite of potential problems of using molecular clock estimates, the divergence times obtained might provide a crude estimate of the relative order of events isolating the major haplotype lineages of *P. sylveirae* in the Brazilian Atlantic Forest.

6 CONCLUSION

Our findings shed light on the phylogeography of *Protonectarina sylveirae* and addressed more information towards the understanding of the complex pattern of diversification for the enormous biodiversity in Brazilian Atlantic Forest.

The strongly structuration found among different populations suggests the presence of barriers which made the genetic flow restrict or even null. The present structure also suggests the occurrence of a complete lineage sorting, the process where populations became monophyletic from each other (AVISE, 2000). Moreover, deep phylogeographical divisions within taxa suggest extensive periods of isolation among some constituent populations of South American species (TURCHETTO-ZOLET *et al.*, 2013).

Morphological differentiation was also found for population in morphometric analysis. Even if not completely congruent with groups evidenced by molecular data, it reveals a distinction between populations of SW groups and the other two groups. No differences in morphological features as color or punctuation were observed for different patterns among populations. In fact, color presents intrapopulation differences. Several studies revealed the presence of deep intraspecific phylogeographical structure with multiple lineages (phylogroups) (TURCHETTO-ZOLET *et al.*, 2013). This could be indicative of cryptic species, that is, morphologically indistinguishable (BICKFORD *et al.*, 2007). However, genetic distance among populations or haplogroups are not big enough and we believe that this kind of affirmation requires a more accurate study.

From the haplotype network it was possible to observe three main haplogroups (Central Southeast - CS, South-West - SW and Northeast groups - NE) although it was not possible to determine the ancestral haplotype. Demographic analysis shows stability for most populations, indicating that they had remained constant throughout its evolutionary history. However, signs of recent expansion for TRI and CANO. Genetic and geographic patterns found were also strongly influenced by the eusocial way of life.

The dating analysis indicates an origin and divergence of haplogroups starting from middle to late Miocene, a time older than Plio-Pleistocene, refuting the idea of refuges. Notwithstanding, according to Turchetto-Zolet *et al.* (2013) there is no clear overall pattern indicating that in South America, most species originated during the Pleistocene, as predicted by the refuge theory. However, it probably modeled the current distribution of species. It was not possible to attribute specifically events to the divergence of lineages.

Corridors of forest reaching the current Caatinga region and the increasing of dryness during the Miocene, isolating populations from wet areas are potential explanations for the divergence among lineages. What seems evident is that not only Pleistocene Milankovitch cycles but also earlier orogenic events (i.e. volcanic, tectonic, mountain uplift, alteration in drainage systems) occurring during the Miocene and Pliocene probably played an important role in the population divergence of South American species (RULL, 2011) and also for *P. sylevirae*.

This work indicates that *Protonectarina sylveirae* presents an early evolutionary history in Neotropical region, consistent with events that occurred in the tertiary, particularly the isolation of forest areas caused by increasing in dryness starting from this period, which led to strong genetic structuring observed. There has been little phylogeographic work on social wasps, being this study pioneer in attempt to provide a more complete picture of the evolution and diversification of paper wasps and helping adding more pieces to solve the jigsaw of historical diversification of biota in South America.

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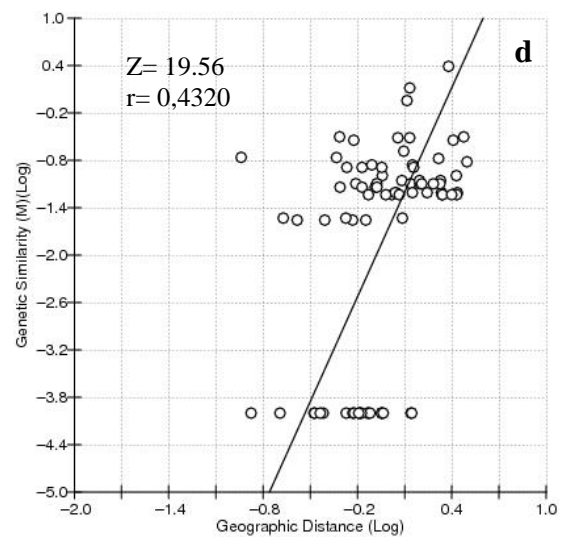
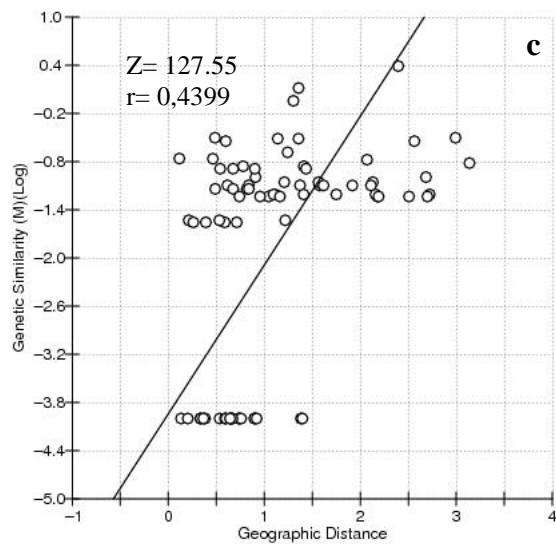
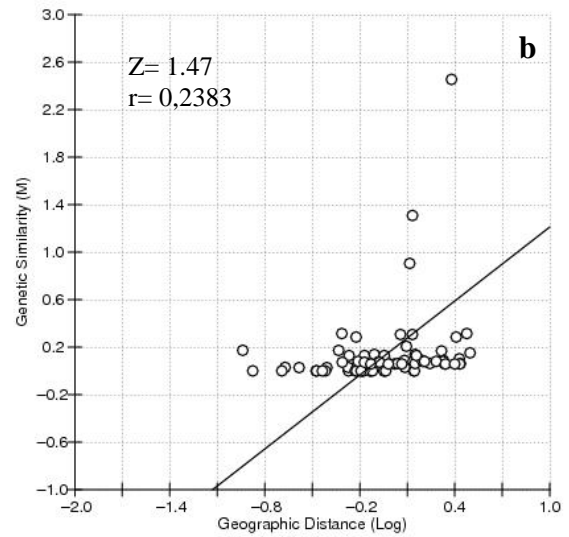
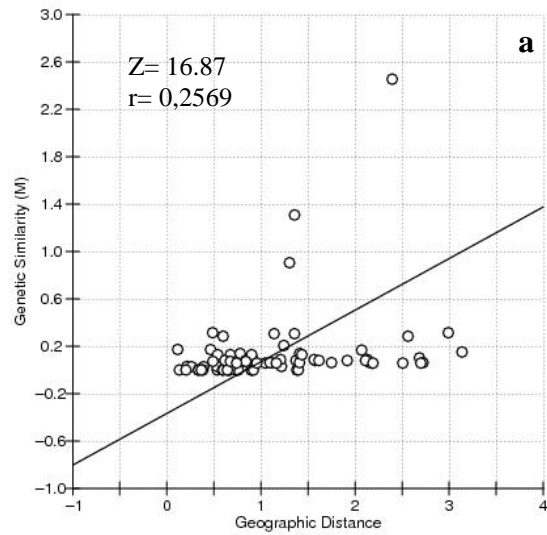
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APPENDIX A - Used PCR conditions for the amplification of gene loci sampled for this study.

Gene	Fragment	Step 1	Step 2			N° of cycles (step 2)	Step 3
		Initial denaturation	Denaturation	Annealing	Extention		Final extention
EF-1 α	2	94°C/4 min.	94°C/1min.	54°C/1min.	72°C/1.5min.	35	72°C/7 min.
12S	---	94°C/2 min.	94°C/30s.	49°C/15s.	68°C/30s.	35	72°C/7 min.
16S	----	94°C/2 min.	94°C/15s.	47°C/15s.	72°C/15s.	40	72°C/6 min.
COI	1	94°C/2 min.	94°C/30s.	48°C/30s.	72°C/45s.	40	72°C/7
	2	94°C/5 min.	94°C/45s.	51°C/45s.	72°C/1,5min.	35	min.

APPENDIX C - Graphics of Mantel test. Lines represents RMA regression. Z, Pearson's correlation coefficient between matrix; r, Pearson's statistics. P value were significant for b, c and d, indicating no correlation (value of r is close to 0).



APPENDIX D – Chronogram with divergence times generated by BEAST. Divergence times between clades are annotated on each node and standard deviations [95% of high posterior density (HPD)] are represented by the blue bars.

