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RESEARCH ARTICLE

Neither barriers nor refugia explain genetic structure in a major biogeographic break: phylogeography of praying mantises in the Brazilian Atlantic Forest

Bernardo F. Santos[a], Marcus V. Scherrer[b,c] and Ana Carolina Loss[b]

[a]Department of Entomology, National Museum of Natural History, Washington, DC, USA; [b]Universidade Federal do Espírito Santo, Vitória, ES, Brazil; [c]Instituto Federal do Espírito Santo, Santa Teresa, ES, Brazil

ABSTRACT

The Atlantic Forest is one of the world's top biodiversity hotspots, but the diversification processes of its biota are still poorly known, with competing models attributing dominant roles to either Quaternary climatic changes or geographic barriers. Many studies identify the Doce river as a major phylogeographic break, but the reasons for this phenomenon are highly debated. Here we test the predictions of the refugial and barrier models for a common species of praying mantis, *Miobantia fuscata*, focusing in the areas immediately south and north of the Doce river. Our analyses show high intraspecific genetic diversity, deep coalescence times and no evidence for recent population expansion. Phylogeographic structure is inconsistent with a refugial hypothesis. Significant gene flow between northern and southern populations also conflicts with a strong role for geographic barriers. This study highlights the need for considering invertebrate taxa to infer recent landscape changes, and points towards a more complex picture of genetic diversification in the Atlantic Forest.

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KEYWORDS
Doce river; central corridor; *Miobantia*; Mantodea; Thespidae

Introduction

Climate and habitat fluctuations through the Late Quaternary have been one of the main frameworks for investigating current patterns of diversity and endemism (Hewitt 2000; Carnaval et al. 2009). In the tropics, much attention has been paid to the potential role of Pleistocene refugia models, also known as forest refuge hypothesis (FRH, *sensu* Leite et al. 2016). The FRH originally proposed that successive episodes of forest fragmentation through glacial cycles led to isolation and consequent speciation in the Amazon Forest (Haffer 1969). Although initially proposed in the context of a specific biome and time period, the FRH was eventually invoked to explain biodiversity patterns in other time periods (Haffer 1997) and ecosystems, including South America's Atlantic Forest (Prance 1982).

The Atlantic Forest (AF) is one of the top biodiversity hotspots in the world (Myers et al. 2000; Laurance 2009). In spite of this, the processes that generated its biodiversity remain relatively poorly known. A current model aiming to explain the phylogeographic patterns and increase of genetic diversity in this ecosystem is the Carnaval–Moritz (CM) model of forest dynamics (Carnaval and Moritz 2008). This may be considered a revitalized version of the FRH based on the application of paleodistribution models for species and habitats. These models predict a large area of historical forest stability (refugial) in the central corridor of the AF, while most of the coastal areas south of the Doce river experienced significant reduction within the cool dry period of the Last Glacial Maximum (LGM), around 21,000 years ago. Rather than considering refugia as drivers of allopatric speciation, the CM model emphasizes their role in sheltering populations during glacial periods, and associates the high current genetic diversity within species and endemism in the AF with these historically stable areas (Carnaval et al. 2009).

There are a number of predictions embedded in the CM model: (1) refugial areas should present higher genetic diversity than non-refugial regions, because of long-term persistence of populations in these areas; (2) non-refugial areas should present genetic signature of recent population expansion, reflecting colonization after the Last Glacial Maximum; (3) strong phylogeographic structure should be expected between refugia, reflecting long term isolation between populations in these areas.

Paleopalynological evidence supports the idea that large portions of what is today the southern AF were occupied by savannah-like environments during the Last Glacial Maximum, while central and northern areas remained covered by forest (Behling 1997, 1998, 2003; Ledru et al. 2005). However, other paleobotanical studies have shown that humid forests had persisted throughout glacial cycles (Colinvaux et al. 1996; Pessenda et al. 2009). A recent study has shown that, by incorporating the area of the emerged continental shelf on the Atlantic Forest during glacial periods, paleoclimatic

CONTACT Bernardo F. Santos santosbe@si.edu Department of Entomology, National Museum of Natural History, 10th and Constitution Ave. NW, Washington, DC 20560-0165, USA

Supplemental data for this article can be accessed here.

models show evidence for *less* fragmentation during the Last Glacial Maximum (Leite et al. 2016). This so-called 'Atlantis Forest hypothesis' suggests that topographic heterogeneity from southern Atlantic Forest associated with available area from emerged continental shelf favored a cold yet humid weather in this region. Thus, one should not expect to find genetic signature of recent expansion in southern species, once forest fragmentation may not have been relevant. Therefore, the role of refugial areas in shaping current biodiversity patterns is still controversial.

Empirical tests of the CM model have variously supported its predictions (Costa 2003; Pellegrino et al. 2005; de Moraes-Barros et al. 2006; Grazziotin et al. 2006; Cabanne et al. 2007; Carnaval et al. 2009; Fitzpatrick et al. 2009; Resende et al. 2010; Ventura et al. 2012) or rejected them (e.g. Zamudio and Greene 1997; Leite 2003; Thomé et al. 2010; Batalha-Filho et al. 2012; Amaro et al. 2012; Thomé et al. 2014; Brunes et al. 2015; Cardoso et al. 2015; Cabanne et al. 2016; Leite et al. 2016). Some of the latter studies suggest that geographical barriers may play a more prominent role in shaping diversification in the Atlantic Forest (Thomé et al. 2010; Amaro et al. 2012; Thomé et al. 2014; Brunes et al. 2015). Barrier hypotheses entail diverse genetic predictions from those expected under the CM model, including older divergence times, interruption of gene flow coinciding with putative barriers and smoother demographic changes across time.

In many of the species studied so far, the observed phylogeographic structure shows a north-south division pattern, often having the Doce river as a phylogeographic break (see Costa and Leite 2012 for a review, and Carnaval et al. 2014 for an aggregate analysis of 25 vertebrate taxa). In some cases, it has been suggested that the river itself may represent a barrier to the gene flow between northern and southern regions (Pellegrino et al. 2005; Cabanne et al. 2008; Cazé et al. 2016), while other authors have emphasized the distinct climatic regimes and floristic composition found in the AF to the north of the Doce river (Carnaval et al. 2014; Leite et al. 2016). Some studies, however, have found no particular influence of this barrier in shaping phylogeographic patterns (e.g. Menezes et al. 2017). Hence, understanding the role of this particular biogeographic contact zone may illuminate other aspects of the AF diversification.

Herein, we test the predictions of both refugial and barrier models of genetic diversification focusing specifically in the purported phylogeographic break represented by the Doce river. As a suitable study system, we selected a species of praying mantises within the genus *Miobantia* Giglio-Tos (Mantodea, Thespidae, Miobantiinae), a group of small, brown-coloured mantises endemic to the Atlantic Forest (Scherrer 2014). The species *Miobantia fuscata* Giglio-Tos (Figure 1), specifically, is distributed through the Central Corridor of the Atlantic Forest (Scherrer 2014) to the north and south of the Doce river. In this area, they represent one of the most conspicuous praying mantis taxa, often found in leaf litter or in plants close to the ground. All females are wingless, whereas the males are winged and very active, often captured by active search or by flight interception traps (Scherrer 2014).

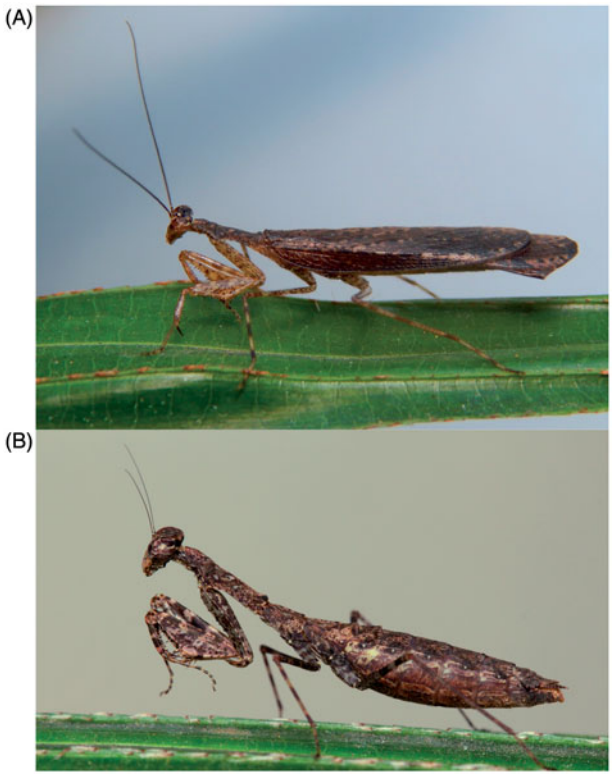

Figure 1. Live specimens of *Miobantia fuscata*. (A) male, winged; (B) female, wingless.

Materials and methods

A total of 102 specimens of *Miobantia fuscata* from 25 localities in the Atlantic Forest (Figure 2) were sampled. Samples are provenient from the central region of the AF, particularly around the Doce river, where the break between two climatic domains in the AF is expected to be found (Carnaval et al. 2014).

Eighty sequences generated by Scherrer (2014) for the mitochondrial gene *cytochrome oxidase I* (COI) were combined with newly generated ones using the similar protocols, except by the following. Genomic DNA was isolated from the sample tissue using standard protocols for the DNeasy Blood and Tissue Kit (Qiagen, Düsseldorf, Germany). Polymerase chain reactions were performed in 25 μL using 2.0 μL of template DNA, 1.0 μL of each primer at 10 nM, 21.0 μL of water and illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare Life Sciences, Little Chalfont, UK). Thermocycler programs were similar to the ones in Scherrer (2014) except with a 47 °C annealing temperature and 68 °C extension temperature. Samples were purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA), and sequencing was performed in a 96-well ABI PrismTM 3730xl automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Multiple sequence alignment for this dataset is relatively trivial and was performed using MUSCLE (Edgar 2004), under default parameters, and sequences were trimmed to 602 base pairs (bp) to include only data available for all or almost all species. All generated sequences have been deposited in GenBank (Supplementary Appendix 1).

Phylogenetic analyses were performed using Bayesian inference in BEAST v1.8.4 (Drummond et al. 2012) and

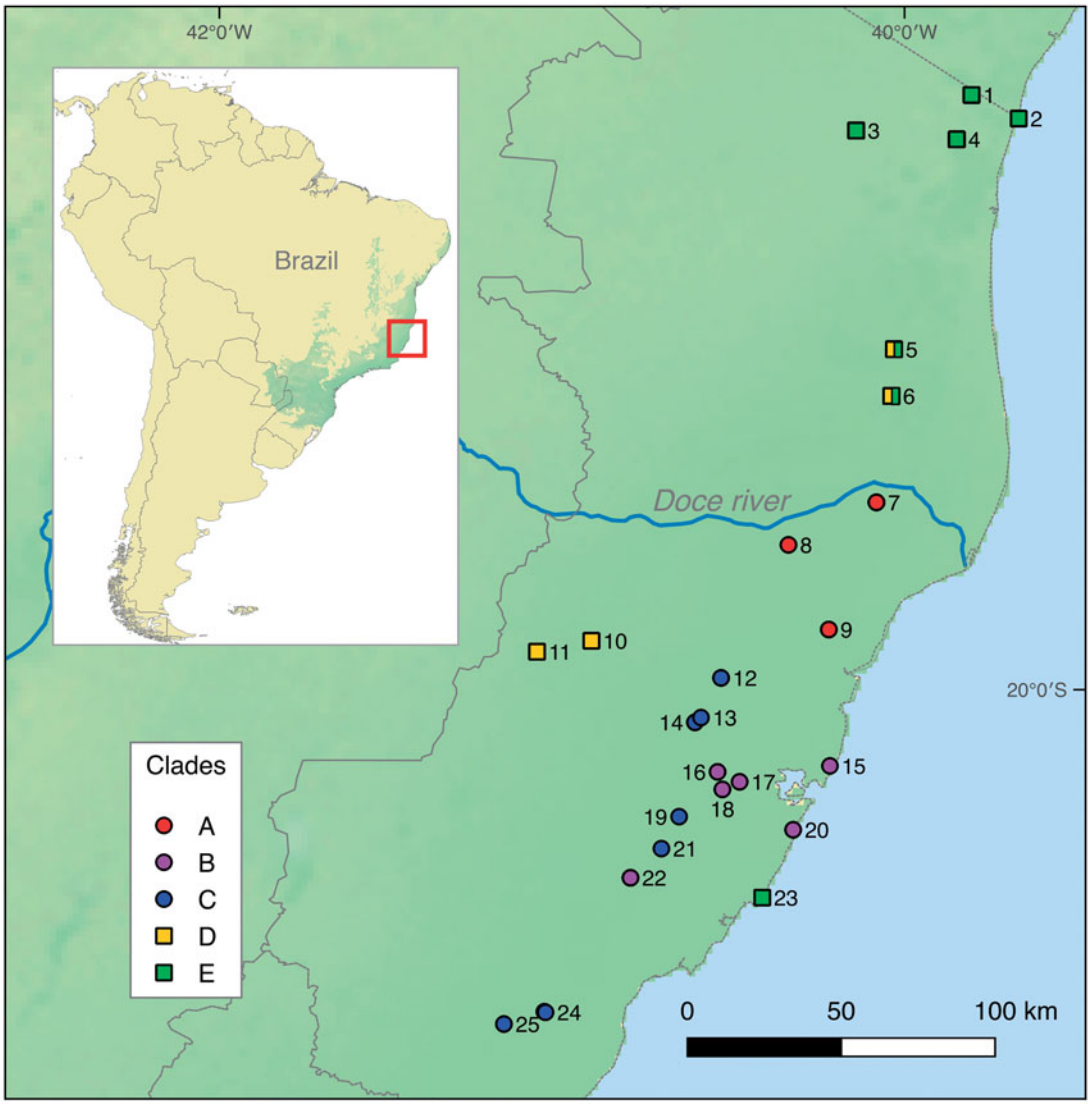

Figure 2. Map showing collection sites in the present study. The Atlantic Forest is indicated in green in the top left detail, with the sampled localities colour coded as per clades recovered in phylogenetic analyses (see Figure 3). Numbers correspond to localities as shown in Supplementary Appendix 1. The map was generated using the software QGIS v2.14 (www.qgis.org).

maximum-likelihood RaxML-HPC2 (Stamatakis 2006). Specimens from *Miobantia aptera* Giglio-Tos and an undescribed species of *Miobantia* were used as outgroup taxa. The best-fit model of molecular evolution for each codon position was estimated using PartitionFinder2 (Lanfear et al. 2017), under the Akaike Information Criterion, as follows: GTR + G for the first position; HKY + G for the second position and GTR + G for the third. Models that estimated a proportion of invariant sites ('+I' parameter) were not considered to avoid the risk of overparametrization (Mayrose et al. 2005; Stamatakis 2006). Bayesian analyses were run for 6.5×10^8 generations, with trees sampled every 10^3 generations. LogCombiner, from the BEAST package, was used to resample trees at a lower frequency (5×10^3), resulting in 1.3×10^5 trees. Tracer v1.6 (Rambaut et al. 2014) was used to verify stationarity of the Markov chains through ESS values. A maximum credibility tree was generated in TreeAnnotator 1.8.4 (Drummond and Rambaut 2007), with the first 10% states discarded as burn-in. All phylogenetic analyses ran through the CIPRES Science Gateway platform (https://www.phylo.org). Trees were graphically visualized and edited with Figtree v 1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/) and Adobe Illustrator CC 2017.

Population genetic metrics were calculated in DnaSP 5.1 (Librado and Rozas 2009), as follows. Due to the difficulty of standard population genetics software to deal with missing data, taxa with ambiguities in variable sites were excluded from the analyses, which were based on 94 sequences from 23 localities. The number of segregating sites (S), nucleotide diversity (π) and theta per site (Θ) were used to estimate genetic diversity in populations north and south of the Doce river, where the phylogeographic rupture between refugial and non-refugial areas was supposed to be. Tajimas' D (Tajima 1989) and Fu's Fs (Fu 1997) were used to test for recent population expansion against a null hypothesis of constant population size. Fu's test was demonstrated to perform better than other similar methods for samples with $n > 20$ (Ramos-Onsins and Rozas 2002). Fst and Nm were calculated to estimate the gene flow between northern and southern populations of *M. fuscata* (Hudson et al. 1992). Relationships

among haplotypes were assessed using median joining networks generated in the software PopArt (Leigh and Bryant 2015) and graphically edited at Adobe Illustrator CC 2017.

A spatial analysis of molecular variance was conducted using the program SAMOVA 2.0 (Dupanloup et al. 2002) to delimit groups of populations that were genetically homogenous, but maximally differentiated from each other. The program uses simulated annealing procedures to seek the best clustering option that can be defined between a given number (K) of groups of populations. The goal was to test whether southern and northern populations would be naturally grouped together when K was set to delimiting two groups of populations, or whether there was significant geographic signal in the groups of populations. Analyses were based on 100 simulated annealing steps with K increasing from 2 to 10. Genetic pairwise distances were calculated in MEGA5 (Tamura et al. 2011) using the evolutionary model Kimura 2-parameter (Kimura 1980).

In the absence of closely related fossil and homologous COI sequences to calibrate molecular clock analyses, we estimated a substitution rate for COI in *Miobantia fuscata* using phylogenetic relationships for Mantodea and secondary calibration dates from Svenson and Whiting (2009). We used 53 COI sequences, including 18 from *Miobantia* and 35 from other Mantodea species covering 32 genera. The best-fit model of molecular evolution determined by PartionFinder2 was GTR + G for each codon position. We used an uncorrelated lognormal relaxed molecular clock in BEAST and Yule tree prior with five calibration points with normal prior distribution. We forced monophyly of clades to reflect tree topology according to Svenson and Whiting (2009) and our results from the phylogenetic analysis of *Miobantia*. Only highly supported clades were considered (details on calibration points and phylogenetic topology in Supplementary Appendix 2). We ran two independent replicates. LogCombiner was used to combine files after burn-in into one file with 8×10^7 states, sampling one tree every 8×10^3 generations.

We used coalescent Bayesian Skyline Plot (BSP; Drummond et al. 2005), implemented in BEAST, to infer demography history for each northern and southern populations, applying a strict clock with a COI substitution rate of 1.058% per million years per site estimated as described above. The best-fit models of molecular evolution for each codon position for northern population were TRN + G for the first position, HKY for the second position and GTR + G for the third. For southern populations, codon position models were, respectively, TRN + G, GTR + G and TRN + G. Analyses ran for 10^8 generations, with trees sampled every 10^4 generations. Bayesian Skylines for each population were reconstructed using Tracer.

Results

Genetic diversity, phylogenetic relationships and phylogeographic structure

The phylogenetic tree revealed a considerable degree of phylogeographic structure between northern and southern areas (Figure 3). Both Bayesian Inference and maximum-likelihood analyses recovered five stable and well supported main clades, but the relationships among them are equivocal, with low posterior probabilities and bootstrap values. Three of these main clades comprise only southern populations (Clades A, B, C); one is divided in a north and a south clade, both with relatively low genetic divergence (Clade D); and one last clade is represented by one single southern population sister to a subclade containing the majority of the northern populations (Clade E). In spite of the uncertainty of the relationships among major lineages within *M. fuscata*, the results show clearly that both northern and southern areas represent non-monophyletic groups. When considering clades that show both northern and southern distributions (Clades D and E), those populations are recovered as reciprocally monophyletic inside each clade. Such structure is concordant with geographic distance between sampled localities and therefore does not suggest a phylogeographic break. The best-fit phylogenetic hypotheses in both the Bayesian Inference (maximum credibility tree) and ML (maximum log-likelihood tree) are congruent with the scenario of northern populations diverging from a southern lineage, while in no part of the tree are the southern populations recovered as a subset of a major northern background.

The edited COI sequences resulted in a matrix that produced 52 haplotypes and showed high genetic diversity within *M. fuscata*, with up to 18.3% of pairwise distance between specimens. This variation is probably overestimated due to saturation, since ~10% of the sites were multiallelic. The genetic distance between clades varied from 12.2% (between Clades B and C) up to 16.3% (between Clades C and D). Within clades, the genetic distance was smaller, ranging from 3.4% (Clade B) to 9.9% (Clade C). Since clades show some geographic overlap (Figure 2), this overall genetic variation can be only partially linked to geographic distance, as shown by some of the clades recovered being represented by both southern and northern populations. In fact, the most genetically divergent specimens were collected circa 120 km away from each other (localities 11 and 21, both south of the Doce river). Within northern and southern populations, the variation was often small, though many localities were represented by multiple haplotypes. The genetic diversity was somewhat higher in southern populations than in the northern ones (Table 1). When measured as the number of segregating sites (S) or nucleotide diversity (π), the difference between southern and northern populations was considerable. Northern populations showed more haplotypes than southern ones (31 versus 21), but the overall haplotype diversity was almost identical (0.961 versus 0.967).

As in the phylogenetic trees, the unrooted haplotype network (Figure 4) shows an incomplete split between northern and southern populations, with three southern haplotypes (sites 10, 11 and 23) clearly closer to the set of northern haplotypes.

The spatial analysis of molecular variance conducted with SAMOVA failed to identify groups of southern and northern populations as genetic clusters. When the program was set to delimit only two groups of populations (K = 2), a number of southern populations were grouped together with the northern ones, against a small group of central-southern populations (Supplementary Appendix 4A). Increasing K to define

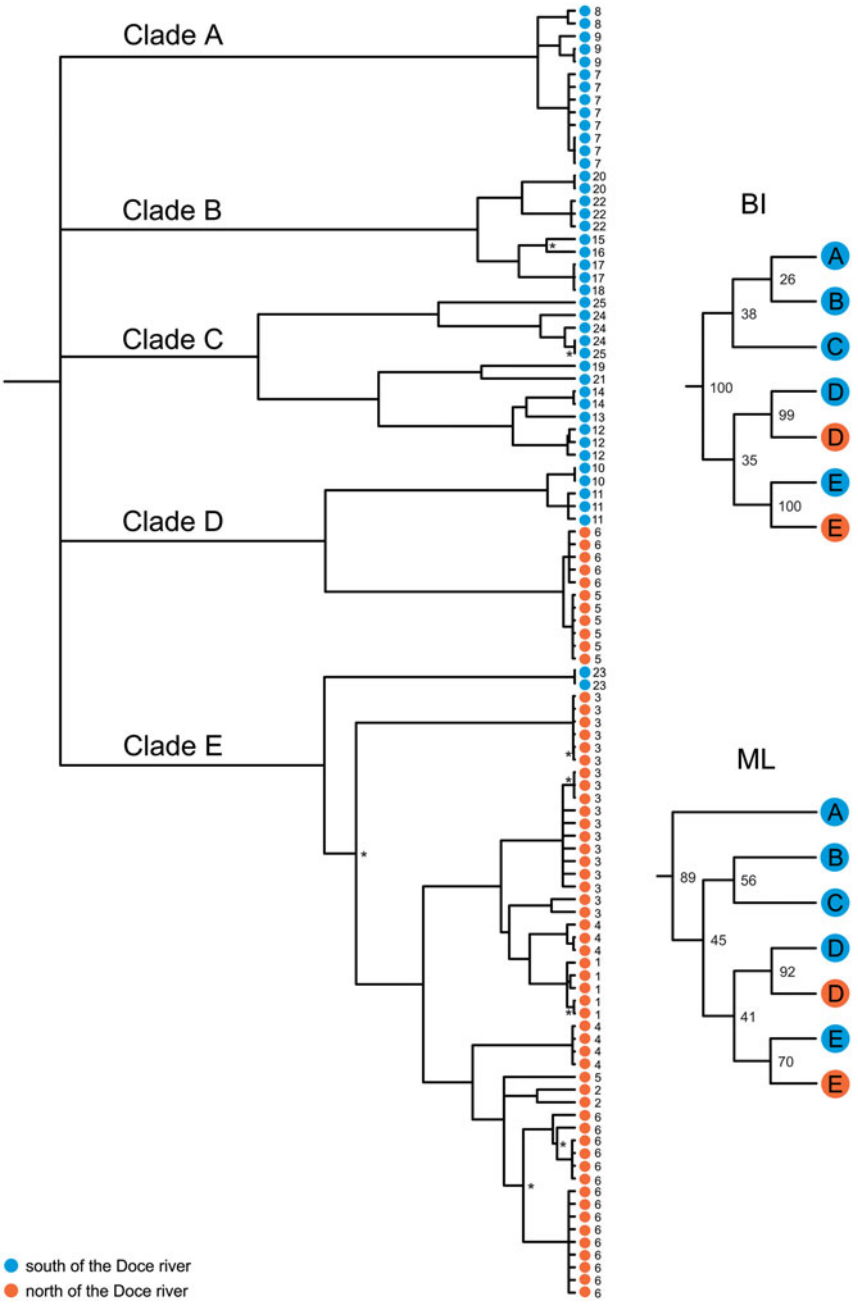

Figure 3. Tree topology recovered for phylogenetic analyses using Bayesian Inference. All nodes with less than 70% posterior probability were collapsed. Nodes without indication of support value have posterior probability of 95% or higher. Nodes marked with an asterisk have posterior probability between 70 and 95%. In the detail, topologies recovered under Bayesian Inference and maximum likelihood for the relationship among the five main clades. Blue and orange circles represent sites at the south and north of the Doce river, respectively.

Table 1. Population genetic summary metrics.

Area	n	loc	S	π	h (Hd)	*D* (*p* value)	*Fs* (*p* value)
North (refuge)	57	6	150	8.003	31 (0.961)	0.76888 (.836)	4.131 (.901)
South (non-refuge)	37	17	181	10.310	21 (0.967)	0.38939 (.716)	7.912 (.990)
All samples	94	23	213	10.575	52 (0.981)	0.40437 (.732)	2.542 (.817)

n: number of specimens in the dataset; loc: number of localities; *S*: number of segregating sites; π: nucleotide diversity; h: number of haplotypes; Hd: haplotypes diversity; *D*: Tajima's *D*; *Fs*: Fu's *Fs*. Metrics calculated separately for northern and southern populations and for northern and southern groups as a whole.

three or more groups of populations did not show particularly strong geographic signal (Supplementary Appendix 4B). These results indicate that northern and southern populations do not form homogeneous groups.

Historical demographic change

Neutrality tests (Table 1) detected no sign of recent population expansion in any of the analyzed groups ($p > .7$). Our BSP analyses showed a sign of relatively recent population

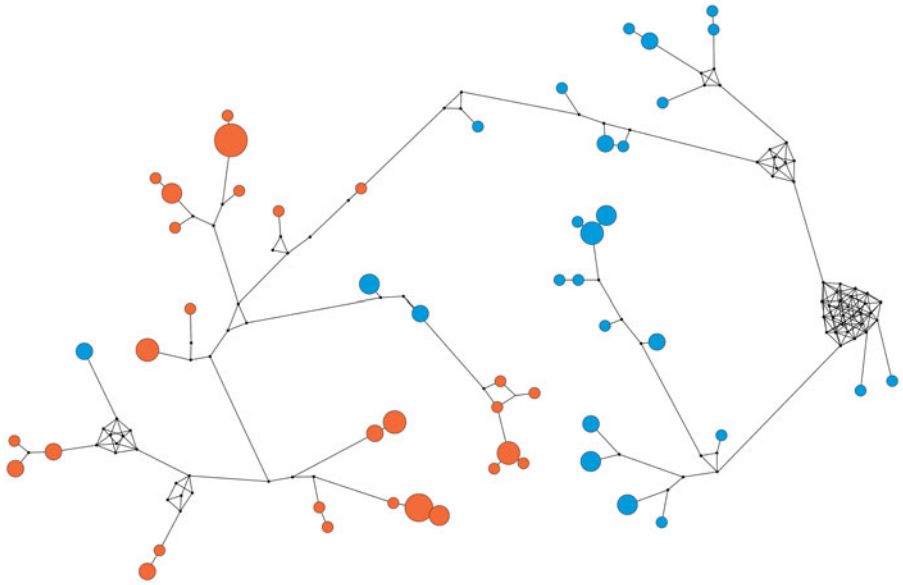

Figure 4. Haplotype network for *Miobantia fuscata*. Blue and orange circles represent sites at the south and the north of the Doce river, respectively. The size of the circles is proportional to the number of individuals with a given haplotype (minimum 1, maximum 9), and the length of the connecting lines is proportional to the number of mutations separating each haplotype.

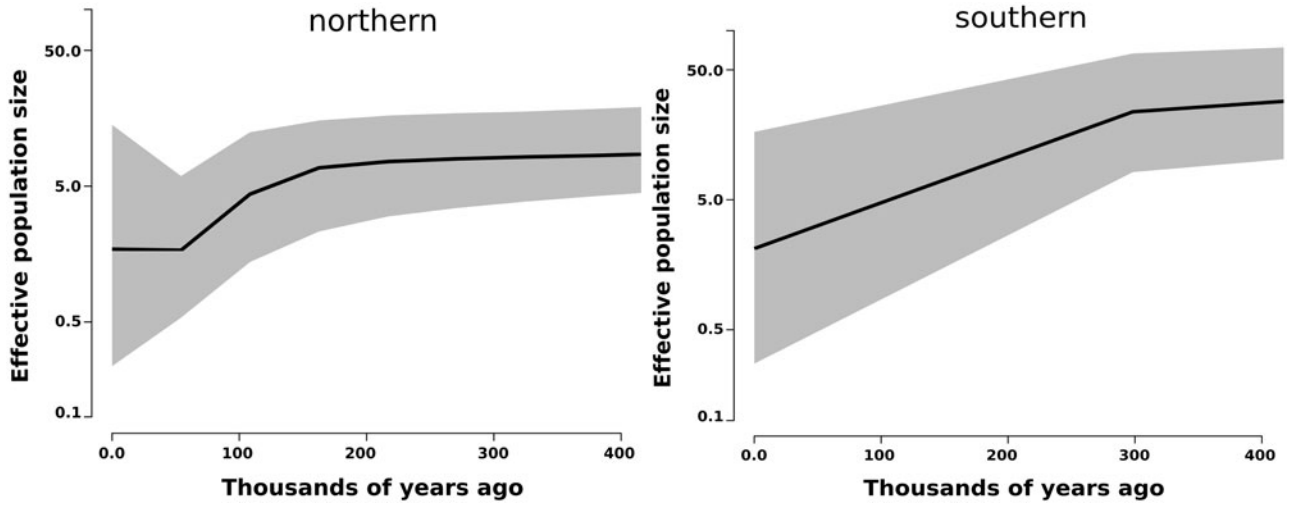

Figure 5. Results from the Bayesian Skyline Plot showing changes in demographic sizes through time for northern (left) and southern (right) populations. The black lines show median values for population sizes over time, with the shaded areas indicating the 95% confidence intervals.

decrease in both southern and northern populations, but at different times (Figure 5). For northern populations, demographic decline started at around 150–50 kya, followed by stability in population size until present. On the other hand, decrease in southern population is older, starting at 300 kya but lasting until present.

Divergence dating analyses (Supplementary Appendix 3) point to an age of 35.43–56.08 Mya for the most recent common ancestor of all *M. fuscata* (mean 45.67, median 45.31). Given the rates of genetic variation for the species, the calculated substitution rate for COI was of 0.55–1.67% per million years per site (mean 1.080%, median 1.058%), which is reasonably close to recorded substitution rates for mitochondrial loci in insects (1.25–1.50% in Farrell 2001). These divergence dates are too old to indicate recent, Quaternary population changes, but the obtained evolution rates were used to calibrate the Bayesian Skyline Plots.

Gene flow between northern and southern populations of *M. fuscata* was low, but not negligible (Fst = 0.27; Nm = 0.66). Some degree of gene flow was also observed among the population of the various clades within *M. fuscata* (Fst = 0.60; Nm = 0.34).

Discussion

Genetic diversity and evolution rates

The remarkable levels of intraspecific genetic variation found in *M. fuscata* (up to 18.3% divergence) may seem surprising, considering for example the purported 3% divergence often proposed to diagnose species using COI sequences (Hebert et al. 2003). However, several other studies have found comparable rates of intraspecific divergence for insects, including up to 30.8% for cockroaches (Che et al. 2017), a group closely

related to praying mantises; 31.15% for thrips (Rebijith et al. 2014) and 21.8% for mosquitoes (Wang et al. 2012). Considering the continuous morphological variation observed in *M. fuscata* and its restrict geographic range (Scherrer 2014), this somewhat remarkable rate of intraspecific divergence seem to be due to old divergence time rather than to the presence of multiple cryptic species.

The idea of older divergence dates for *M. fuscata* is corroborated by our molecular clock analyses, although its results may need to be interpreted with caution. There is considerable criticism on the use of secondary estimations of evolution rates to calibrate divergence dating, since they already embed an error rate, which is carried over to the dating estimation itself (Schenk 2016). However, in the absence of reliable fossils that could be applied to our dataset (see Materials and methods section), we consider that using such estimations to provide an evolution rate for COI in *M. fuscata* is still more precise than using published rates of evolution for insects as a whole, as many studies often do (see Papadopoulou et al. 2010 for discussion on this).

In light of those caveats, the dates for the most recent common ancestor of all *M. fuscata* (35.43–56.08 Mya) should be considered as tentative. While such an ancient divergence for a single species may seem unrealistic, many groups of praying mantises are known to have ancient divergence times. The family Thespidae as a whole date from 120 Mya (Svenson and Whiting 2009), and a recent study on a Caribbean radiation estimated the breakup between two sister genera to be circa 75 Mya (Svenson and Rodrigues 2017). At the same time, the divergence dates estimated by our analyses produced a substitution rate for COI that is fairly congruent with known mitochondrial rates for insects as a whole (see Farrell 2001).

Evidence for competing models

Although our analyses revealed a considerable degree of phylogeographic structure between northern and southern areas, the observed pattern does not match the predictions of the Carnaval-Moritz model of Quaternary refugia, which is contradicted by the following evidence: (1) higher genetic diversity in southern than among northern populations; (2) no traces of recent population expansion in southern (non-refugial) populations; (3) the phylogeographic structure, though considerable, does not fit the expectation of having southern populations nested within a northern background.

Several studies had previously identified a distinction between northern and southern components in the Atlantic Forest; in most of these studies, the faunistic or phylogeographic break occurred around the Doce river (Costa and Leite 2012; Carnaval et al. 2014). In some studies, other river systems also appeared to be associated with phylogeographic breaks (e.g. Costa et al. 2000; Pellegrino et al. 2005; Cazé et al. 2016), which led Pellegrino et al. (2005) to suggest that the role of rivers as barriers could be an important driver of genetic diversification and endemism at the Atlantic Forest. This 'riverine barrier hypothesis' has been addressed for many animal groups in the Amazon (Wallace 1852; Ayers and Clutton-Brock 1992; Patton and da Silva 1998; Gascon et al. 2000; Patton et al. 2000). However, the importance of rivers as speciation drivers in the Amazon ultimately lost credit due to conflicting evidence and to the labiality of river courses in the area (Kalliola et al. 1993; Symula et al. 2003; Gascon et al. 2000). In contrast, Pellegrino et al. (2005) suggest that the Atlantic Forest area is geologically more stable and less likely to have undergone major river course shifts in the recent past.

For *M. fuscata*, a barrier model seems to be a somewhat better fit to the observed patterns of genetic diversity than the CM model. Such a scenario predicts older divergence times (confirmed) and smoother demographic changes (potentially true). Yet, our demographic analyses are in agreement with the 'Atlantis Forest Hypothesis' (Leite et al. 2016) as they show no sign of recent demographic expansion in southern populations, suggesting forest permanence during Quaternary glaciations in this areas. The relative structure of northern and southern populations shown both in the phylogenetic tree (Figure 3) and haplotype network (Figure 4) points to some level of differentiation between these groups of populations. However, the moderate values for Fst and Nm suggest that there is some degree of gene flow between these areas, with approximately one migrant every 1.5 generations. In addition, our SAMOVA analyses suggest that northern and southern populations do not form homogeneous groups, as it would be expected under long term isolation.

In both 'riverine barriers' and Quaternary refugia scenarios, dispersal ability would play an important role; indeed, species with low dispersal ability are often considered ideal to study past population dynamics (Tonini et al. 2013; Carnaval et al. 2014). Winged insects are often good dispersers, as they can be caught in air currents and elevated thousands of meters high (Glick 1939). However, species of *Miobantia* represent a very particular case due to their sex dimorphism. Because all females are wingless and strongly associated with forest leaf litter (Terra 1995 and pers. obs.), their dispersal ability is limited, which is reflected in the mitochondrial genome. Further studies adding nuclear markers can be used to test if the increased dispersal ability of males will uncover a different phylogeographic scenario.

Conclusions

For the praying mantis *M. fuscata*, COI sequences show high intraspecific genetic diversity, deep coalescence times and no evidence for recent population expansion. Some phylogeographic structure was observed between populations to the south and north of the Doce river, but the results are inconsistent with a quaternary refugia model. Significant gene flow and lack of homogeneous variance between northern and southern populations also conflict with the idea of a strong role for the Doce river as a geographic barrier. That implies that, for *Miobantia*, recent landscape changes did not have a major role in shaping genetic diversity and endemism. Considering that the vast majority of existing studies focus on vertebrate taxa, we highlight the need for considering invertebrate taxa when using phylogeography to infer recent landscape change and its consequences to population dynamics. Additional studies with additional species and a

more extensive geographic coverage will provide a better understanding of the phylogeographic structure of these praying mantises at the Atlantic Forest.

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Disclosure statement

The authors have identified no conflict of interest to declare.

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