# High genetic variability in a small toad from the Brazilian Atlantic Forest

# Alta variabilidade genética num pequeno sapo da Mata Atlântica brasileira

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## ABSTRACT

The fragmentation of the Atlantic Forest is one of the main causes of habitat loss in this important global biodiversity hotspot. Amphibians are an integral part of this biodiversity and are the most threatened group of vertebrates on the planet, with some species declining mainly due to habitat loss; therefore, they are an important parameter to understand the effects of fragmentation. One of the least known aspects of this process is how the surrounding matrix fragments influence frog diversity and gene flow among these forest remnants. Moreover, few studies have analyzed genetic variability of

populations. Frogs are key targets for such studies because of their role as bioindicators. This study aimed to determine whether a matrix with predominance of coffee plantations, sugar plantations or pasture influence the genetic diversity of *Rhinella ornata* estimated by analyzing the *D-loop* region of mitochondrial DNA. The results showed that not all tested matrices restricted genetic diversity of this toad, which showed little tendency for population structure even between the most distant fragments tested (102 km).

**Keywords:** amphibians; *D-loop*; mtDNA; *Rhinella ornata*; spatial genetic

**RESUMO**
A fragmentação da Mata Atlântica é uma das principais causas da perda de habitat deste importante *hotspot* da biodiversidade mundial. Os anfíbios representam uma parcela importante dessa biodiversidade e são o grupo de vertebrados mais ameaçado do planeta, com várias espécies em declínio decorrente principalmente da perda de habitat. Assim, torna-se importante entender melhor os efeitos dessa fragmentação e um dos aspectos menos conhecidos deste processo é como o tipo de matriz no entorno dos fragmentos influencia sua diversidade, mas também o fluxo dela entre esses remanescentes florestais. Além disso, são raros os trabalhos que reúnem esse tipo de análise à variabilidade genética das populações. Os anuros são alvos-chave para tais estudos em virtude de sua capacidade como bioindicadores. Esse estudo teve como objetivo verificar se matrizes com predomínio de cafezais, canaviais ou pastagens influenciam a diversidade genética de *Rhinella ornata* estimada pela análise da região *D-loop* do DNA mitocondrial. Os resultados mostraram que nenhum tipo de matriz restringiu a diversidade genética deste sapo, que apresentou pouca tendência à estruturação populacional e evidências de fluxo gênico entre vários dos fragmentos testados, inclusive os mais distantes a 102 km.

**Palavras-chave:** anuros, *D-loop*, mtDNA, *Rhinella ornata*, genética espacial.

## 1 INTRODUCTION

The Atlantic Forest is one of the world's biodiversity hotspots (Myers et al. 2000), extremely rich in biodiversity biomes, with high rates of endemism, but also very threatened. Deforestation resulting from the expansion of agricultural frontiers has led to the fragmentation of these forest ecosystems, limiting them to small patches or isolated fragments (Pimm and Raven 2000). The persistence of the species in these forest remnants depends on several factors, but one aspect that is considered fundamental and yet remains very misunderstood is how the quality of the matrix changes the environment surrounding the fragment. Depending on the use of land in the matrix, it can increase or decrease connectivity among habitat patches and this will be reflected in the absence or non-movement of wildlife (Fahrig and Merriam 1985; Hanski and Gilpin 1991; Ricketts 2001). In these altered landscapes,

some remnants may provide refuge for certain species (Arruda et al. 2011), but the matrices can affect the genetics of residing populations there by interrupting gene flow, and generating population bottlenecks and/or inbreeding. In order to better understand and then reverse this process, it is important to evaluate the effects that habitat fragmentation have on the diversity and genetic structure of the species (Frankham, Ballou and Briscoe 2009), seeking to implement more effective conservation measures.

In addition to intensifying habitat loss, fragmentation is also considered a major cause decline of global amphibian. These animals are currently recognized as one of the groups most at risk for extinction on the planet (Beebee and Griffiths 2005; Stuart et al. 2004) and its decline are likely to accelerate in the twenty-first century (Hof et al. 2011). About 30% of frog species are at risk of disappearing in the coming years (IUCN 2015).

Amphibians with aquatic larvae and associated forests are considered more susceptible to disconnection of forest fragments because they are often forced to cross an inhospitable matrix to arrive at breeding sites (Becker et al. 2010). One of these species typically associated with the Atlantic Forest biome is *Rhinella ornata*. This small toad was chosen as the target of this study, since it seems to be susceptible to negative impacts of fragmentation, especially in smaller fragments (Dixo et al. 2009) and there was a need to verify whether this susceptibility also applies to the matrix. Moreover, this was the most abundant species on the effects of matrix type on amphibian diversity in some of the same forest remnants in this study (D'Anunciação et al. 2013).

An important tool in such studies is mitochondrial gene sequences, largely used in the reconstruction of pedigrees and genetic variability in the description of species (Moritz, Dowling, and Brown 1987). Studying mitochondrial DNA (mtDNA) has advantages over using other portions of the genome as high replacement rate, maternal inheritance and lack of recombination (Gemmell, Metcalf, and Allendorf 2004). mtDNA has a controlling or non-coding region, known as the *D-loop*, which seems to control replication and transcription of the mtDNA region. Analyses of mtDNA control regions have provided a high resolution of intraspecific genetic structure in a variety of taxa (Avise 1994). Furthermore, one study has reported that these sequences are more sensitive to genetic drift and population bottlenecks (Moritz 1994).

This region of the mtDNA was used as a molecular tool in this study to test a gap not explored by previous works (D'Anunciação et al. 2013; Dixo et al. 2009) to check if three predominant and different types of matrices influence the intra and interpopulational genetic variability of an extremely abundant amphibian in forest fragments of the Atlantic Forest.

## 2 MATERIALS AND METHODS

### 2.1 STUDY AREA AND ANIMAL SPECIE

The study was conducted in nine fragments of semideciduous forests in two areas in the South of Minas Gerais State (southeastern Brazil). Eight of these fragments are in the area of the municipalities of Alfenas, Areado and Serrania. The region is transitional area of contact between Cerrado and Atlantic Forest, with an average altitude of 880 m, average annual temperature of 23°C and average annual rainfall of 1600 mm, with two well-defined seasons: dry and cold winters and hot and humid summer (Drummond et al 2005). The eight selected fragments fulfilled three prerequisites: 1) Minimum of 75% in the area with a predominant matrix (coffee, sugar cane or pasture), 2) an area between 15 and 100 ha and 3) minimum distance of 500 m between fragments to ensure relative independence.

The ninth fragment (Figure 1) was considered a control area and comprises the Nova Baden State Park, PNB (21 ° 56 '29.52 "S 45 º 19' 5.91" W, 214.47 hectare), located in the municipality of Lambari, about 100 km from the eight other fragments. It is a mountainous region with altitudes of 900-1300 m, average annual rainfall of 1500 mm and average temperatures of 18°C (Sturaro and da Silva 2010). Unlike the other fragments, PNB is larger and also a conservation unit inserted in an area around which ensures continuity with other green areas of a minimum of 1000 ha of continuous preserved forest.

*Rhinella ornata* is a frog species endemic to the Atlantic Forest, considered a generalist by some authors and able to traverse agricultural matrices between forest fragments. Still, it seems likely to be affected by the negative impacts of fragmentation, especially in smaller fragments (Dixo et al. 2009). Samples of PNB were collected from 2007 to 2009 in sporadic trips and those from the eight fragments between 2011 and 2012, all under a 1934-1 IBAMA license.

Specimens were collected with pitfall traps consisting each of four buckets of 30L and taken in moistened plastic bags to be deposited in the Herpetological

Collection Alfred Russel Wallace (CHARW), Federal University of Alfenas (UNIFAL-MG). In the laboratory, frogs were first euthanized (Cortez, Suárez-Mayorga and López-López 2006), and then liver samples were removed mand preserved in absolute alcohol prior to freezing to be deposited in the Tissue Collection Alfred Russel Wallace (CHARW). The rest of the animal was fixed in 10% formalin and preserved in 70% alcohol, according to Calleffo (2002).

## 2.2 LABORATORY PROTOCOL

We extracted DNA from liver tissue of 106 individuals of *R. ornata* according to the DNA extraction kit EZ-10 Spin Genomic DNA Minipreps Animal (Bio Basic, Toronto, Canada). The quality and quantity of extracted DNA was determined by NanoDrop Spectrophotometer 2000. For amplification, we used Invitrogen reagents according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, US). We amplified fragments of the mtDNA control region using primers ControlWrev-L and ControlP-H (Goebel, Donnelly, and Atz 1999). The PCR amplification conditions were: initial denaturation at 96 ° C for 5 min, followed by 40 cycles of denaturation at 94 ° C for 30 s, annealing at 60 ° C for 45 s, and extension at 72 ° C for 45 sec, followed by a final extension at 72 ° C for 10 min. PCR products were purified using the Exonuclease I and SAP enzymes (Fermentas, Vilnius, Lithuania) which remove the unincorporated dNTPs and primers for subsequent sequencing. The sequencing was done in an automatic sequencer model 3130xl (Applied Biosystems Carlsbad, CA, US) and the sequences obtained were aligned in MEGA version 5.1 (Tamura et al. 2011). All sequences were deposited in GenBank (Acession in KF974575 - KF974680).

## 2.3 GENETIC ANALYSIS OF POPULATIONS

This study investigated the polymorphism of DNA (haplotype and nucleotide diversity) globally and within sampling areas, as well as the estimated differences between populations using DnaSP.

## *2.4 SPATIAL ANALYSIS*

To test the correlation between genetic and geographic distances, the Mantel test (Bonnet and Van De Peer 2002) was performed in IBD v. 1:52 software (Bohonak 2002). Other statistical analyses such as the number of haplotypes in each location

(isolated fragments and the control area), analysis of haplotype diversity and nucleotide diversity were performed in a BioStat 5.0 software (Ayres et al 2007) program. The PAST program v. 1.88 (Hammer, Harper and Ryan 2001) was used to implement the non-metric multidimensional scaling (NMDS) analysis of pairwise FST values, allowing for visualizing the genetic relationships among the nine sampled fragments. Analysis of molecular variance AMOVA (Excoffier, Smouse, and Quattro 1992) was performed in ARLEQUIN v3.11 software (Excoffier, Laval, and Schneider 2005). MEGA v. 5.1 software (Tamura et al. 2011) was used to construct the phylogenetic tree by the Neighbor-joining and Maximum Likelihood methods, using 1000 replicates. The chances of barriers to gene flow were identified by a Barrier v.22 software (Manni, Guérard, and Heyer 2004), according to a declining balance of genetic distances. While the number of barriers has to be set by the user in this program, we defined a consensus quantity, comparing the hypotheses data from pairwise FST data (Table 1). When more than half of the relationships between pairs of samples involved did not sustain the proposed barrier, the same was not implemented. As the chances of a barrier are proposed based on genetic distances in descending order, the first proposal barrier is more likely to occur than the second, which, in turn, is most likely the third and so on. When a hypothetical barrier was not confirmed by the majority of pairwise FST values, the other less likely barriers were not tested.

## 3 RESULTS

### 3.1 GENETIC DIVERSITY OF POPULATIONS

The mtDNA control region of 106 individuals of *Rhinella ornata* was amplified and sequenced resulting in a 666-base-pair long fragment. A gap was detected at position 474 from the 5' end in 29 individuals. According to Table M1, the absolute number of haplotypes for the population ranged from three (P3 fragment and the control area) to 6 (P1). The amount of polymorphic sites ranged from 15 (PNB) to 28 (P1). The number of migrants (Nm) was 1.20. We perform the calculation of measures of connectivity between pairs of location (pairwise FST) totaling 36 combinations of populations, and 17 pairs were significantly correlated. The data of pairwise FST ranged from 0.1667 to 0.4577, with the highest value among the fragments C1 and PNB (Table 1).

The results of neutrality tests were statistically significant, with values of

Tajima's D = 2.679 (p <0.05), and D & Fu * = 2.651 (p <0:02). The multimodal curve (Figure 2) indicates balancing selection.

The network haplotype was constructed at the level of 92% of the limit of connection. We found 10 distinct haplotypes, six of them shared among fragments immersed in different matrix types and four unique haplotypes, each from a single fragment: H3 (sugar cane matrix), H5 (coffee matrix), H8 (pasture matrix) and H10 (sugar cane matrix) (Figure 3 and Table M1).

## 3.2 SPATIAL ANALYSIS

There was no relationship between genetic and geographic distances in the Mantel test (r = 0.2189, p = 0.8010), thus suggesting *R. ornata* is not isolated by geographic distance, even in the control area (PNB), which is about 100 km from the other eight fragments (Figure 4). Regarding the number of haplotypes in each location (isolated fragments and the control area) there was no statistically significant difference (p = 0.277). The same occurred in diversity analysis of haplotypes (p 176 = 0.502) and nucleotide diversity (p = 0.874).

The analysis of multidimensional scaling (Figure 5) confirmed results of the Mantel test, indicating relative genetic similarity between geographically distant samples (such as PNB & S2 and P1 & P3, for example), in addition to non-segregation of fragments according to the type of matrix (sugar cane, coffee or pasture) or the control area.

The analysis of molecular variance (AMOVA, Table 2) also supported the same conclusions, showing great variation in population structure (FST) and moderate to large differences in these populations within groups (FSC). Therefore, although some genetic differentiation between some pairs of fragments that might indicate structure (Table 1), the general mean difference between the groups (FCT) was not significant. The AMOVA showed that genetic variations within populations were mainly responsible for total genetic variability. No evidence of complete structure involving any type of matrix or the control area was observed. The nearest of isolation by distance was the tendency of structuring between the control area and all fragments of coffee, pasture and cane fragments (S1). However, two fragments of sugar cane showed no significant structuring with PNB, as can also be seen in Table 1.

Phylogenetic analysis of all of the individuals also confirmed the lack of

segregation among fragments of different matrix types, as well as individuals in the control area (PNB), which were all mixed. Both the neighbor-joining algorithm as well as Maximum Likelihood showed the same results using 1000 replicates (Figure 6).

Given these results, there were only hypotheses of barriers for tendency of population structuring among some combinations of pairs of sampled fragments (Table 1). Each barrier proposed by the Barrier v.22 software (Manni, Guérard, and Heyer 2004) was compared pairwise FST values (Table 1). If more than half of the pairwise FST values between the supposedly separate samples also indicated breakage of gene flow, the hypothesis of barrier was accepted and then we tested the following hypothesis. Thus, the barrier "A" has been proposed and implemented (Figure 7), but in the second barrier ("B") there was no support from most pairwise FST values and then this was not implemented. Barrier "A" coincides in part with the hypothesis of isolation by distance; it separates the control area (PNB), 100 km away from the other fragments. However, results from two of the fragments surrounded by sugar cane (S2 and S3) did not corroborate such interruption of gene flow (Table 1) and showed to be genetically similar to the control area.

## 4 DISCUSSION

As we observed, the distance between the fragments and the predominant type of surrounding matrix did not unrestrictedly prevent gene flow between sampled populations of *R. ornata*, maintaining stable genetic variability among these populations. These populations are not structured, but there is a possibility for a distance barrier, which interrupts the flow mainly between the fragments and the control area. However, this hypothesis is not applicable to two fragments of sugar cane (S2 and S3). These two fragments showed genetic similarity to the control area, 102 km distant.

Arruda and colleagues (2011) obtained similar results to our study, detecting no influence of the matrix on the genetic diversity of the same toad genera. This study compared microsatellite loci *Rhinella schneideri* fragments with sugar cane and fragments inserted into pasture matrix and found the population structure and isolation by distance or separate samples up to 138 km, thus considered a species with wide dispersal ability. Although studies are still preliminary, the results observed to date indicate that this kind of frog has great dispersal ability associated with more generalist habits. This may explain the high intensity of population expansion and

spatial distribution of *Rhinella marina* introduced in Australia, for example (Estoup et al. 2010; Llewelyn et al. 2010). Therefore, more studies on the influence of the matrix on genetic diversity are needed. Many studies on the effects of matrix type (Bernarde and Macedo 2008; D'Anunciação et al. 2013; Gustafson and Gardner 1996; Isaacs Cubides and Urbina Cardona 2011; Neckel-Oliveira 2004; Neckel-Oliveira and Gascon 2006; Pardini et al. 2005; Pineda and Halffter 2004; Santos-Barrera and Urbina-Cardona 2011; Silva, Martins, and Rossa-Feres 2011; Urbina-Cardona, Olivares-Pérez, and Reynoso 2006) do not include genetic data and most genetic studies on the effects of fragmentation ignore the matrix (Dixo et al. 2009; Hitchings and Beebee 1998; Moore et al. 2011; Pröhl and Krug 2013).

The present study sought to fill this gap, being a pioneer in the analysis of the influence of fragmentation using the control region (*D-loop*) of mtDNA as a molecular marker of forest populations of an amphibian inserted into three predominant types of matrix: sugar cane, pasture and coffee. The other study addressing the same species and the same molecular tool (Dixo et al. 2009) did not test the influence of the matrix type, only the size and distance between the fragments.

Another aspect that is rare in studies on fragmentation, but was done in our study and also by Dixo and et al. (2009), is a comparison with a control area. In our case, this area is located within a conservation unit, which has a wide range of preserved forest (214.47 hectares within the limits of the park, but more than 1,000 continuous ha was added to the environment). Although it is a relatively distant control area (about 100 km), surprisingly, there were also indications of possible gene flow of PNB with at least two of the sampled fragments.

The expectation that gene flow between fragments was interrupted by certain types of land use has not been confirmed in our results. Although the results of pairwise FST point to structuring among some of the sampled populations, no genetic differences associated with specific type of matrix (AMOVA and phylogenetic tree), or in relation to geographical distance (Mantel test) and the value of Nm obtained was greater that 1 (1.20). According to Wright (1931), only values of Nm (gene flow) less than 1 indicate genetic isolation. Added to these findings the hypothesis of barrier coincides with the greatest distance (PNB) and still not fully supported by all fragments. Thus, in general, we conclude that there is gene flow among the majority of the population (detected by Nm and the phylogenetic tree),

but not at the point of homogenizing them, and allowing differentiation (haplotype network, for example). We infer that they share genes, regardless of the surrounding matrix and relative distance of the fragments, but with a small hint of structure in some of them.

Although these findings are consistent with other studies (Arruda et al. 2011; Dixo et al. 2009), they apparently contradict a study (D'Anunciação et al. 2013) with an amphibian community in the same area. This study points out the conditions of the pasture matrix as more conducive to amphibians, especially for *R. ornata*, in relation to the sugar cane matrix. The abundance of this species in the same fragments predominantly surrounded by sugar cane (in the present study) was extremely lower (15) than that found in fragments surrounded by pasture (106). A significantly lower abundance would be expected that might have reflected the observed reduced genetic variability and / or a reduction of gene flow, which was not confirmed. An alternative hypothesis is that these declines are recent and they have not been sufficient to generate negative impacts on the genetic diversity of these populations time. This does not mean, however, that these impacts will not appear after a certain time. A point that may strengthen this hypothesis is that coffee plantations and pastures characterize the predominant land use in this region long ago and just recently sugar cane became an option for cultivation. Regarding fragmentation, species may respond differently to changes in habitat (Ewers and Didham 2006) (Wiens 1994) and most studies in fragmented landscapes occurred on a scale of less than 100 years after the fragmentation time (Ewers and Didham 2006; Watson 2002), which prevents an understanding of their long-term effects (Watson 2003). Even so, we consider the range of 50-90 years sufficient to ensure a balance of diversity patterns (Ewers and Didham 2006; Renjifo 1999).

Another alternative hypothesis is that the decline observed by Annunciação et al. (2013) in populations of *R. ornata* fragments surrounded by sugar cane is not a decline in fact, but just a normal population fluctuation. These authors collected data for two consecutive years, but in the third year, there were population growth or dispersal events in the most affected fragments, these populations may have recovered. Importantly populations tested in our study present an irregular and multimodal distribution curve (Figure 2), different than expected under the hypothesis of recent population expansion or decline, which indicates a relative demographic stability (Rogers and Harpending 1992).

Fluctuations in population size may be more the rule than the exception and many reports of declines may be natural reductions that precede later stages of growth. Such fluctuations may be related to rain, predation, competition, disturbance and other factors that may also influence the dynamics of amphibian populations at different stages of the life cycle (Wilbur 1987). Some studies have shown that these large fluctuations are relatively common in many populations of amphibians (Pechmann et al. 1991; Semlitsch et al 1996).

Another aspect involves the discussion of the *R. ornata* as a generalist or specialist. This discussion was already present in the literature (Araujo and de Almeida-Santos 2013; Ribeiro-Júnior and Bertolucisp 2009), but according to the results of D'Anunciação et al. (2013), the species was affected by the type of matrix (sugar cane) and could therefore be considered sensitive and relative potential as a bioindicator. The results of our study did not find a negative effect of matrix isolation and neither was checked by distance. If this is confirmed, this species could even be favored by fragmentation, since it is extremely abundant and genetically stable in a heavily fragmented landscape. Therefore, *R. ornata* would actually be a generalist species, as advocated by Dixo et al. (2009).

Another way to consider this species as generalist is the possible gene flow among distant populations about 100 km. Amphibians in general are considered animals with low dispersal ability (Berry 2001; Blaustein, Wake, and Sousa 1994; Duellman and Trueb, 1986). These populations according to some authors will be found at most 300-1,000 m from their breeding grounds, depending on the taxon (Crawford and Semlitsch 2007; Pittman, Osbourn, and Semlitsch 2014; Schabetsberger et al. 2004; Sinsch et al. 2012). This dispersal ability, however, has been little tested (Smith and Green 2005) and there are already some indications that some amphibians can cover great distances, especially the Bufonidae family and genus *Rhinella*. *Rhinella marina* introduced in Australia, for example, expands its range at a rate of 50 km/ year (Phillips and Shine 2006); *R. schneideri* presents evidence of genetic connectivity at distances up to 138 km (Arruda et al. 2011) and *R. ornata*, up to 15.8 km (Dixo et al. 2009).

This dispersal capacity need not be fully active or intrinsic to the species. Other mediators can enlarge the displacement of even more sedentary species. On a small-distance scale, floods may be a mechanism to extend this dispersion, carrying egg masses, tadpoles and even adults. Reductions in the frequency of floods have the

ability to limit the spread of amphibians (Wassens et al. 2008). Waterfowl can carry eggs accidentally stuck to their paws (Figuerola and Green 2002), mainly due to a type of spawning bufonids, consisting of hundreds of eggs arranged in a gelatinous cord similar to a beaded necklace. This may be the reason for the success of the family's dispersion. In addition, it is not necessary that individuals roam such extreme physical distances, simply that their genes do. In the Stepping Stones hypothesis (Forman and Godron 1986) the landscape elements facilitate the movement of individuals between fragments in the same landscape so we can infer that individuals with extreme populations need not move the maximum distance to reproduce if their genes end up doing so through successive reproductions of intermediate populations.

This study provides further evidence on the wide dispersal ability of bufonids, but with some differences. The target species here is much smaller than *R.schneideri* (Arruda et al. 2011) and *R. marina* (Phillips and Shine 2006) and it is known that larger species tend to have higher dispersal capacity than smaller ones (Peters 1983). Furthermore, the type of matrix is rarely addressed in studies of fragmentation, especially those including genetic aspects. Likewise, the assumption that the majority of amphibians have meta-population characteristics is rarely tested (Smith and Green 2005).

The metapopulation model takes into account population size, gene flow and the influence of fragmentation, partially dividing the habitat into isolated patches. We thus infer that the distance between the fragments, the degree of insulation and the surrounding matrix type has an influence upon the species restriction in the fragmented areas. But the biology of each species or, for example, their general or specialist character can be crucial in this restriction. Thus, generalist species cannot be affected by fragmentation or may even be benefited by it. In the specific case of *R. ornata* in the present study, the type of matrix and the distance between the fragments are not factors responsible for promoting population structure. Generalist species, however, also have potential bioindicators. Their presence or population growth may indicate negative impacts and when they start to decline, the specialist species are even more endangered.

Even if it is confirmed that the matrix type does not influence the genetic variability of *R. ornata* and other toads, this does not disprove that other landscape

parameters may be responsible for the decline in amphibian populations.

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## Figures and Legends

Figure 1. Study area divided into two landscapes distanced about 100 km. A) Eight forest fragments in the municipalities of Alfenas, Areado and Serrania; C1, C2 fragments in coffee matrix; P1, P2, P3: fragments in pasture matrix; S1, S2, S3: fragments of sugar cane matrix. B) control area in the municipality of Lambari; PNB: Nova Baden State Park.

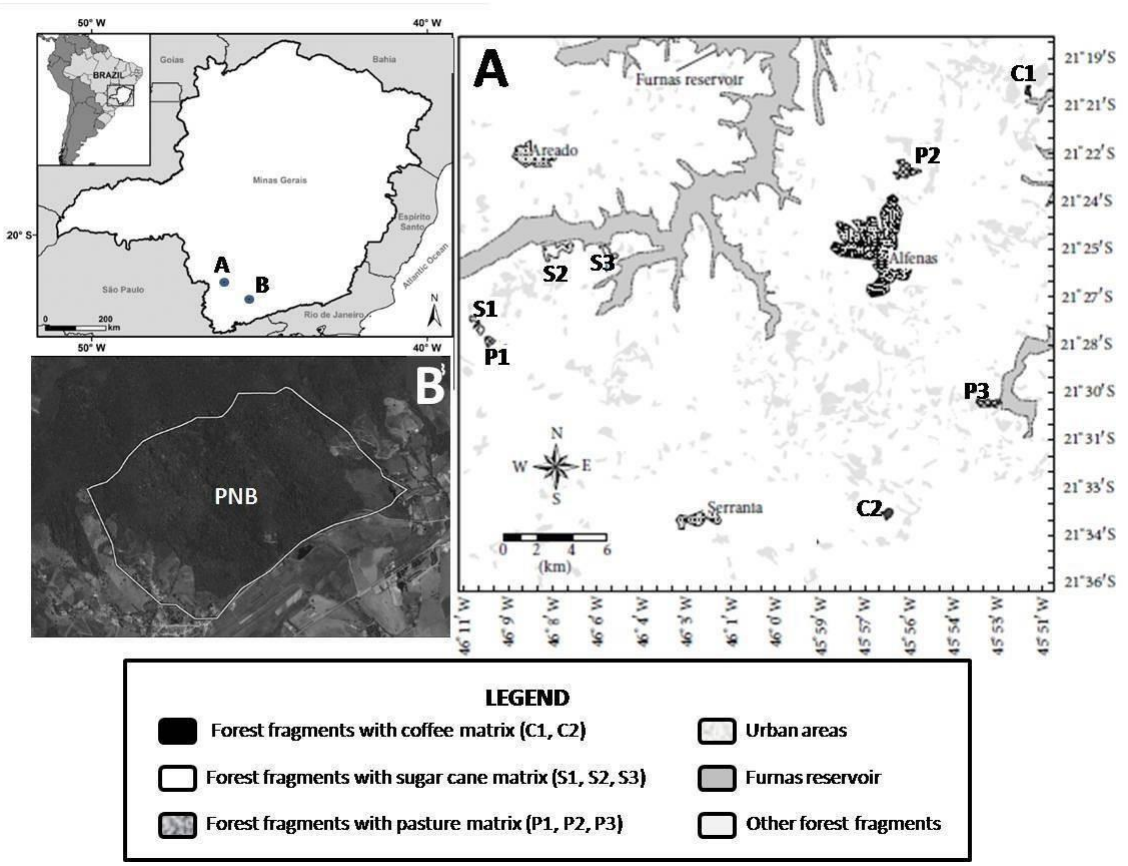

Table 1. Pairwise FST. Estimate of all pairwise combinations of *R. ornata* populations included in the study. Significant values (p <0.05) are indicated in boldface. Pairwise geographic distance (km) are on top.

| | P1 | P2 | P3 | S1 | S2 | S3 | C1 | C2 | PNB |
|---|---|---|---|---|---|---|---|---|---|
| P1 | | 026.09 | 028.47 | 001.78 | 006.44 | 008.79 | 034.06 | 024.73 | 101.41 |
| P2 | **0.1698** | | 014.24 | 026.35 | 020.88 | 017.84 | 009.06 | 020.11 | 088.71 |
| P3 | 0.0496 | **0.1667** | | 029.93 | 026.73 | 023.75 | 018.30 | 009.38 | 075.99 |
| S1 | 0.0060 | -0.0022 | -0.0261 | | 006.39 | 008.80 | 035.13 | 025.84 | 102.92 |
| S2 | **0.2210** | **0.2129** | **0.3736** | **0.3050** | | 003.68 | 029.21 | 025.00 | 101.79 |
| S3 | 0.0134 | 0.0500 | 0.0739 | 0.0305 | -0.0371 | | 025.82 | 021.60 | 098.65 |
| C1 | **0.1755** | 0.0996 | **0.2357** | -0.0250 | **0.4409** | **0.2800** | | 025.85 | 087.26 |
| C2 | 0.0921 | 0.0019 | -0.0032 | -0.1450 | **0.3428** | 0.0732 | 0.0492 | | 076.64 |
| PNB | **0.3404** | **0.1683** | **0.3903** | **0.3434** | 0.0135 | 0.0479 | **0.4577** | **0.3358** | |

Figure 2. Pairwise Differences. Observed and expected fragments of eight sampled (P1, P2, P3, S1, S2, S3, C1, C2) and control area (PNB) of *R. ornata* values indicate multimodal curve.

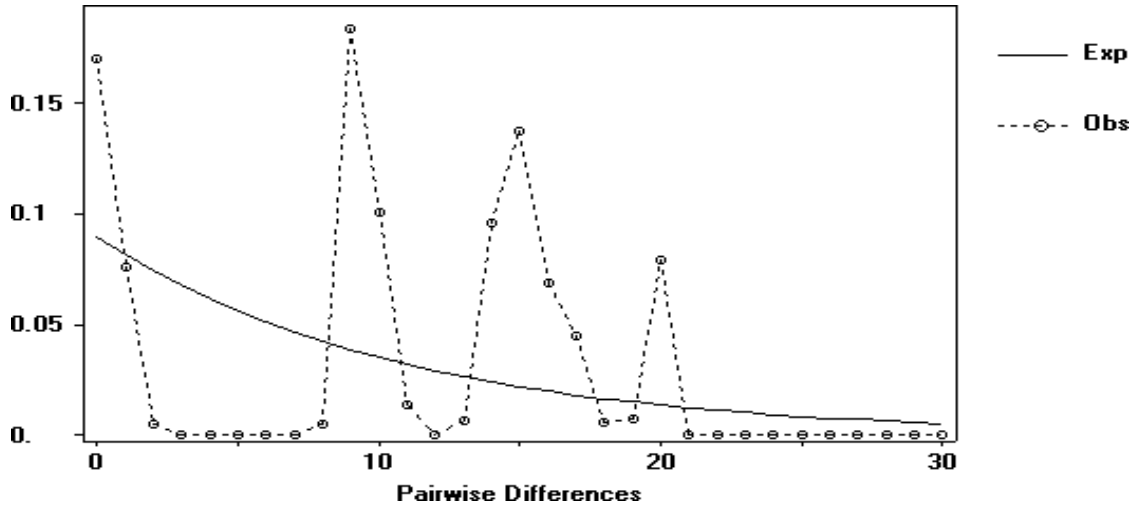

Figure 3. Haplotype network based on the *R. ornata* 666 bp mtDNA control region fragment. Lines connecting haplotypes indicate a difference of a single base pair; the points are represented by steps of mutations. The size of the circles for each haplotype is proportional to its relative frequency in the sample. Green: control area; Yellow: matrix of coffee; Blue: matrix of sugar cane; Red: pasture matrix.

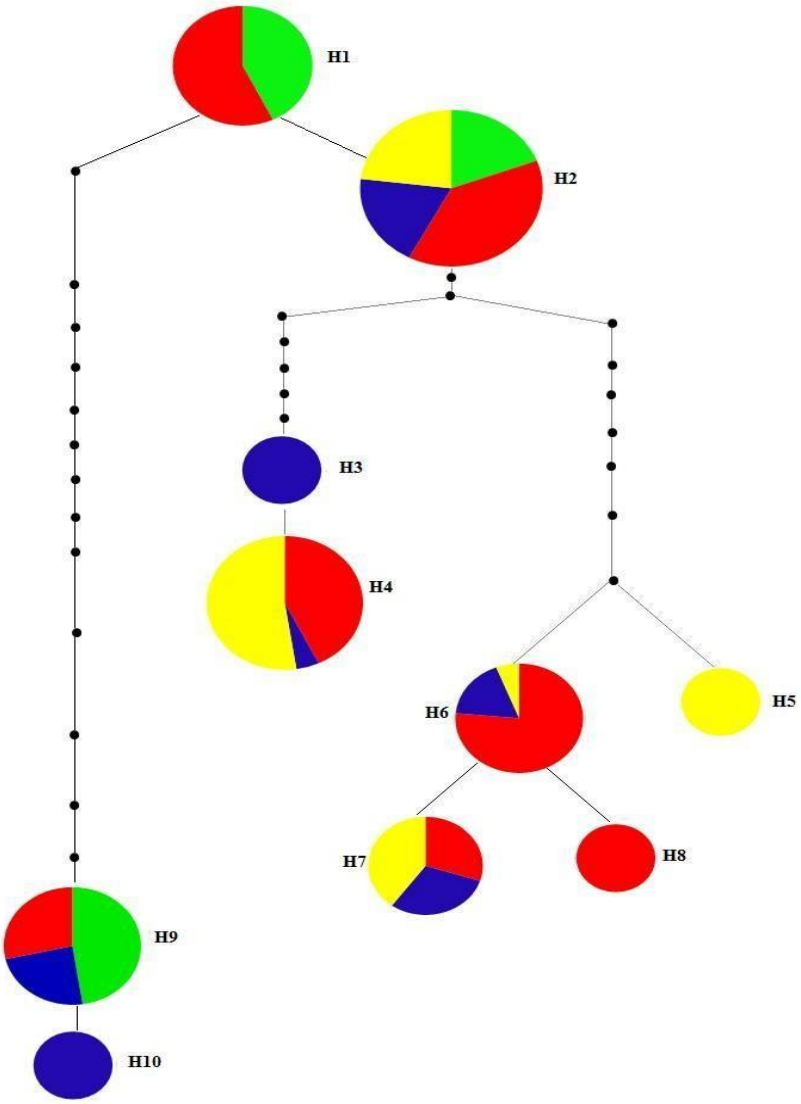

Figure 4. Genetic distance between pairs of fragments as a function of geographic distance. White circles represent relationships only between fragments and black circles, relations involving the control area (PNB).

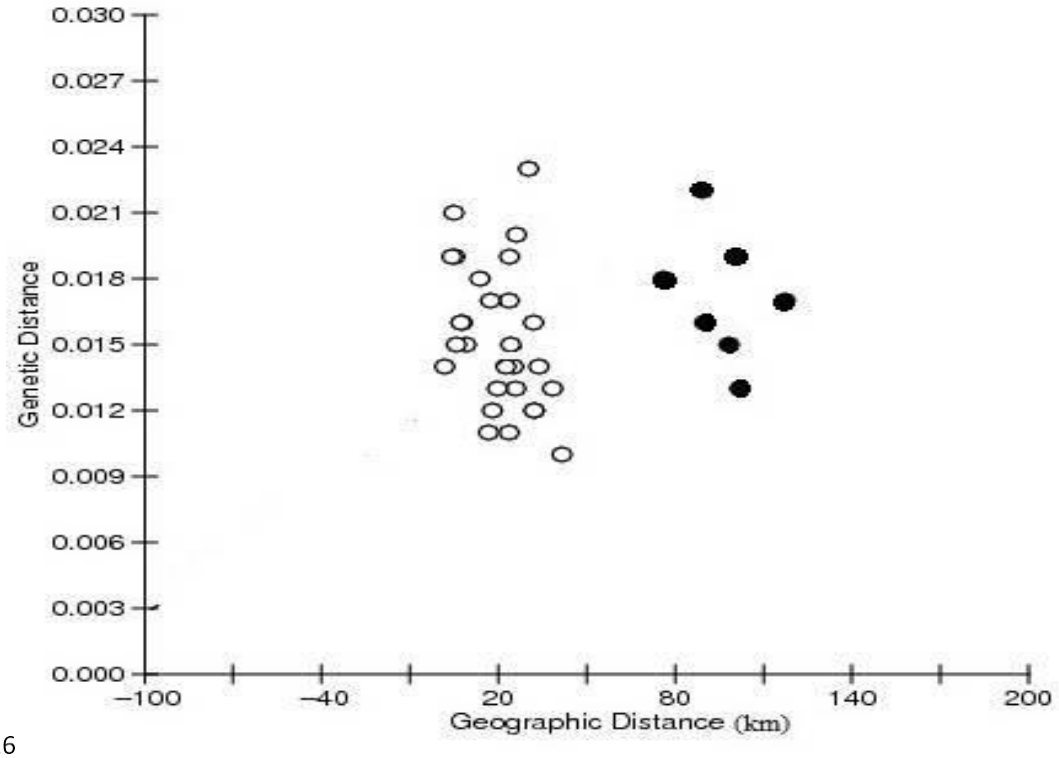


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Figure 5. Graph non-metric multidimensional scaling (NMDS) between axes 1 and 2 from the data of pairwise FST for *R. ornata* of the nine fragments studied. PNB: control area; C1, C2 fragments in coffee matrix; P1, P2, P3: fragments in pasture matrix; S1, S2, S3: sugar cane matrix fragments.

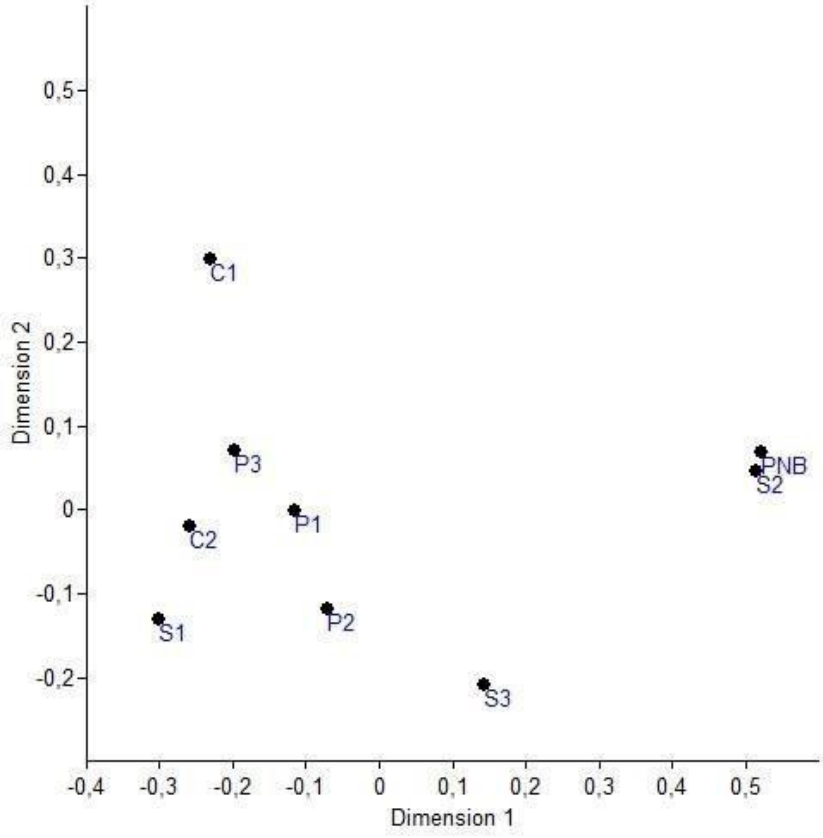


Table 2. Analysis of molecular variance (AMOVA) for genetic similarity of *R. ornata* in different combinations of sampled fragments. PNB: control area; Matrices: P1, P2, P3 (pasture), S1, S2, S3 (sugar), C1, C2 (coffee). Significant values ($p < 0.05$) are indicated in boldface.

| Groups | F values | p-values | %Var |
|---|---|---|---|
| 2groups | $F_{CT}$=0.1482 | 0.1183 | 14.82 |
| **PNB** | **$F_{SC}$=0.1644** | 0 | 14.01 |
| **Matrices** | **$F_{ST}$=0.2882** | 0 | 71.17 |
| 2groups | $F_{CT}$=0.2 | 0.2541 | 12 |
| **PNB** | **$F_{SC}$=0.1844** | 0.0039 | 16.23 |
| **Pasture** | **$F_{ST}$=0.2823** | 0 | 71.77 |
| 2groups | $F_{CT}$=-0.031 | 0.5161 | -3.09 |
| **PNB** | $F_{SC}$=0.1593 | 0.1007 | 16.42 |
| **Sugar** | **$F_{ST}$=0.1332** | 0.0283 | 86.68 |
| 2groups | $F_{CT}$=0.3901 | 0.3314 | 39.02 |
| **PNB** | $F_{SC}$=0.0496 | 0.2766 | 3.02 |
| **Coffee** | **$F_{ST}$=0.4204** | 0 | 57.96 |
| 2groups | $F_{CT}$=-0.016 | 0.5914 | -1.59 |
| **Pasture** | **$F_{SC}$=0.1629** | 0.001 | 16.55 |
| **Sugar** | **$F_{ST}$=0.1496** | 0.001 | 85.04 |
| 2groups | $F_{CT}$=-0.020 | 0.8025 | -2.03 |
| **Pasture** | **$F_{SC}$=0.1477** | 0.0029 | 15.07 |
| **Coffee** | **$F_{ST}$=0.1303** | 0 | 86.97 |
| 2groups | $F_{CT}$=0.1536 | 0.2258 | 15.36 |
| **Sugar** | $F_{SC}$=0.1094 | 0.1124 | 9.26 |
| **Coffee** | **$F_{ST}$=0.2462** | 0.001 | 75.38 |
| 4groups | $F_{CT}$=0.1808 | 0.1808 | 8.51 |
| **PNB** | **$F_{SC}$=0.1501** | 0.0029 | 13.73 |
| **Pasture** | **$F_{ST}$=0.2224** | 0 | 77.76 |
| **Sugar** | | | |
| **Coffee** | | | |

Figure 6. Phylogenetic tree obtained by the method of maximum likelihood (ML), using the HKY model (ln = 1065.237), bootstrap values for 1000 replicates represented in node. The bar indicates number of substitutions/sites. PNB: control area; C1, C2 fragments in coffee matrix; P1, P2, P3: fragments in pasture matrix; S1, S2, S3: fragments of sugar cane matrix.

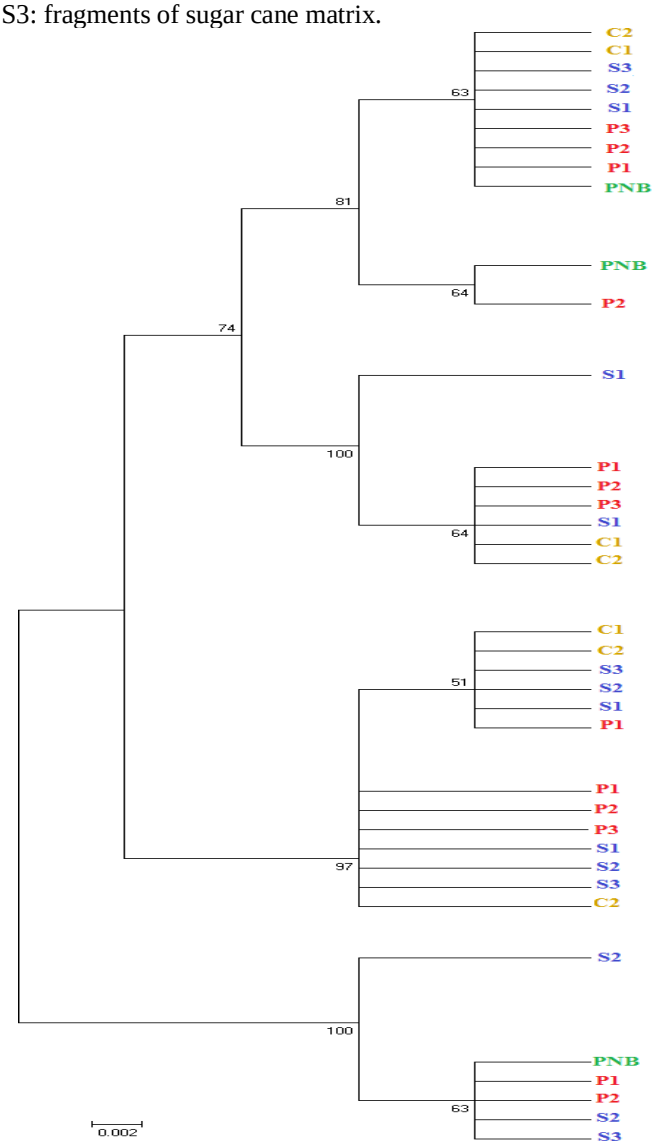
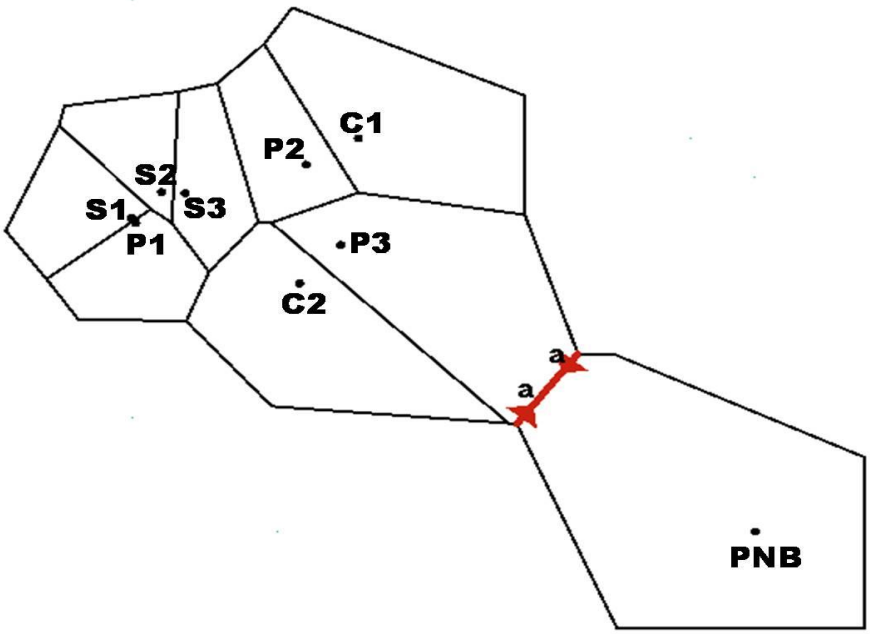


Figure 7. Barrier hypothesis ("a") to gene flow among sampled populations of *R. ornata* (polygons) proposed by the Barrier v.22 spatial analysis program. PNB: control area; C1, C2 fragments in coffee matrix; P1, P2, P3: fragments in pasture matrix; S1, S2, S3: sugar cane matrix fragments



# Supplementary Material

Table M1. Haplotype frequency of the *Rhinella ornata* mtDNA *D-loop* for eight forest fragments sampled in the region and a control area of Atlantic Forest (PNB) in Minas Gerais State (Brazil). Lists of haplotypes (H1-H10), number of individuals analyzed in each location (N), adjusted number of haplotypes (H/N), haplotypes unique to a single population (H), polymorphic sites (P), haplotype diversity (h) and nucleotide diversity (n).

| **Haplotype** | PNB | P1 | P2 | P3 | S1 | S2 | S3 | C1 | C2 |
|---|---|---|---|---|---|---|---|---|---|
| H1 | 0.167 | | 0.267 | | | | | | |
| H2 | 0.278 | 0.067 | 0.133 | 0.467 | 0.333 | 0.125 | 0.333 | 0.133 | 0.500 |
| H3 | | | | | 0.167 | | | | |
| H4 | | 0.200 | 0.333 | 0.0667 | 0.167 | | | 0.600 | 0.250 |
| H5 | | | | | | | | 0.0667 | |
| H6 | | 0.333 | 0.067 | 0.467 | 0.167 | 0.125 | 0.167 | | 0.125 |
| H7 | | 0.200 | | | 0.167 | 0.125 | 0.167 | 0.200 | 0.125 |
| H8 | | 0.067 | | | | | | | |
| H9 | 0.556 | 0.133 | 0.200 | | | 0.500 | 0.333 | | |
| H10 | | | | | | 0.125 | | | |
| H | 3 | 6 | 5 | 3 | 5 | 5 | 4 | 4 | 4 |
| H/N | 0.166 | 0.400 | 0.333 | 0.200 | 0.833 | 0.625 | 0.666 | 0.266 | 0.500 |
| P | 15 | 28 | 27 | 16 | 17 | 22 | 21 | 17 | 17 |
| h | 0.621 | 0.838 | 0.809 | 0.600 | 0.933 | 0.786 | 0.867 | 0.619 | 0.750 |
| n | 0.012 | 0.014 | 0.015 | 0.009 | 0.013 | 0.014 | 0.016 | 0.011 | 0.011 |

## DATA ACCESSIBILITY

DNA sequences data: GenBank accessions KF974575 - KF974680

Moreno-Cotulio.sqn CT94 KF974575

Moreno-Cotulio.sqn CT96 KF974576

Moreno-Cotulio.sqn CT99 KF974577

Moreno-Cotulio.sqn CT103 KF974578

Moreno-Cotulio.sqn CT100 KF974579

Moreno-Cotulio.sqn CT289 KF974580

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Moreno-Cotulio.sqn CT298 KF974588

Moreno-Cotulio.sqn CT104 KF974589

Moreno-Cotulio.sqn CT105 KF974590

Moreno-Cotulio.sqn CT106 KF974591

Moreno-Cotulio.sqn CT107 KF974592

Moreno-Cotulio.sqn CT1153 KF974593

Moreno-Cotulio.sqn CT1154 KF974594

Moreno-Cotulio.sqn CT1155 KF974595

Moreno-Cotulio.sqn CT1156 KF974596

Moreno-Cotulio.sqn CT1898 KF974597

Moreno-Cotulio.sqn CT2102 KF974598

Moreno-Cotulio.sqn CT2121 KF974599

Moreno-Cotulio.sqn CT1157 KF974600

Moreno-Cotulio.sqn CT1970 KF974601

Moreno-Cotulio.sqn CT1971 KF974602

## SAMPLE LOCATION

| Tissue Collection | Fragment | Coordinates |
|---|---|---|
| CT094 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT096 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT099 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT103 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT100 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT289 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT290 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT291 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT292 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |

| | | |
|---|---|---|
| CT293 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT294 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT295 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT297 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT298 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT104 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT105 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT106 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT107 | Nova Baden State Park, PNB | 21º 56’ 29.52” S 45º 19’ 5.91” W |
| CT1153 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1154 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1155 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1156 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1157 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1158 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1159 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1898 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT2102 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT2121 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1970 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1971 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1972 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1985 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1954 | fragment in pasture matrix (P1) | 21º 28’ 15.48” S 46º 9’ 37.03” W |
| CT1219 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT1220 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT1221 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT1223 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT1224 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT1823 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |

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|---|---|---|
| CT1832 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT1834 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT2085 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT2097 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT2098 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT2099 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT2100 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT2101 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT1222 | fragment in pasture matrix (P2) | 21º 22’ 55.25” S 45º 55’ 44.37” W |
| CT2006 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” O |
| CT2007 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” O |
| CT2009 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” O |
| CT2010 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT2038 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT1125 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT1824 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT2188 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT2189 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT1126 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT1127 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT1129 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT1130 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT1131 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT1132 | fragment in pasture matrix (P3) | 21º 30’ 15.43” S 45º 52’ 54.20” W |
| CT1148 | fragment of sugar cane matrix (S1) | 21º 27’ 49.31” S 46º 9’ 56.65” W |
| CT1149 | fragment of sugar cane matrix (S1) | 21º 27’ 49.31” S 46º 9’ 56.65” W |
| CT1850 | fragment of sugar cane matrix (S1) | 21º 27’ 49.31” S 46º 9’ 56.65” W |
| CT1150 | fragment of sugar cane matrix (S1) | 21º 27’ 49.31” S 46º 9’ 56.65” W |
| CT1151 | fragment of sugar cane matrix (S1) | 21º 27’ 49.31” S 46º 9’ 56.65” W |
| CT1152 | fragment of sugar cane matrix (S1) | 21º 27’ 49.31” S 46º 9’ 56.65” W |

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|---|---|---|
| CT1139 | fragment of sugar cane matrix (S2) | 21º 25’ 26.02” S 46º 7’ 30.43” W |
| CT1140 | fragment of sugar cane matrix (S2) | 21º 25’ 26.02” S 46º 7’ 30.43” W |
| CT2184 | fragment of sugar cane matrix (S2) | 21º 25’ 26.02” S 46º 7’ 30.43” W |
| CT2186 | fragment of sugar cane matrix (S2) | 21º 25’ 26.02” S 46º 7’ 30.43” W |
| CT2187 | fragment of sugar cane matrix (S2) | 21º 25’ 26.02” S 46º 7’ 30.43” W |
| CT380 | fragment of sugar cane matrix (S2) | 21º 25’ 26.02” S 46º 7’ 30.43” W |
| CT1141 | fragment of sugar cane matrix (S2) | 21º 25’ 26.02” S 46º 7’ 30.43” W |
| CT1142 | fragment of sugar cane matrix (S2) | 21º 25’ 26.02” S 46º 7’ 30.43” W |
| CT1143 | fragment of sugar cane matrix (S3) | 21º 25’ 31.21” S 46º 5’ 35.77” W |
| CT1144 | fragment of sugar cane matrix (S3) | 21º 25’ 31.21” S 46º 5’ 35.77” W |
| CT1145 | fragment of sugar cane matrix (S3) | 21º 25’ 31.21” S 46º 5’ 35.77” W |
| CT1146 | fragment of sugar cane matrix (S3) | 21º 25’ 31.21” S 46º |
| CT1138 | fragment in coffee matrix (C2) | 21º 33’ 47.04” S 45º 56’ 14.74” W |