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

Alta variabilidade genética num pequeno sapo da Mata Atlântica brasileira

DOI:10.34117/bjdv7n1-433

Recebimento dos originais: 15/12/2020
Aceitação para publicação: 15/01/2021

Bruna Lorencini da Silva
Mestre em Biotecnologia
Instituição: Universidade Federal de Alfenas – UNIFAL-MG
Endereço: Rua Gabriel Monteiro da Silva, 700 – Centro – Alfenas-MG – CEP 37130-001
E-mail: bruna.lorencini.silva@gmail.com

Tereza Cristina Orlando
Doutora em Ciências (Biologia da Relação Patógeno-Hospedeiro)
Instituição: Universidade Federal de Alfenas – UNIFAL-MG
Endereço: Rua Gabriel Monteiro da Silva, 700 – Centro – Alfenas-MG – CEP 37130-001
E-mail: tecrisorlando@gmail.com

Vinícius Xavier Silva
Doutor em Ciências Biológicas – Zoologia
Instituição: Universidade Federal de Alfenas – UNIFAL-MG
Endereço: Rua Gabriel Monteiro da Silva, 700 – Centro – Alfenas-MG – CEP 37130-001
E-mail: vxsilva@gmail.com

Vanessa Roma Moreno Cotulio
Doutora em Ciências Biológicas – Genética
Instituição: Universidade Federal de Alfenas – UNIFAL-MG
Endereço: Rua Gabriel Monteiro da Silva, 700 – Centro – Alfenas-MG – CEP 37130-001
E-mail: biovanessa@gmail.com

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

**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,

**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.
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 ° 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 and 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 (Accession 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 Halfatter 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 than 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.

ACKNOWLEDGEMENTS
This work was supported by FAPEMIG/VALE S.A. (CRA-RDP-00104-10) and an undergraduate fellowship to B.L.S. (PIBICT/FAPEMIG Program 029/2012). We would like to thank Nara Cristina C. P. Barbosa for support with the genetic structure programs. Jim Hesson of mAcademicEnglishSolutions.com revised the English. We thank PPGCA for financial support.
REFERENCES

Araujo, C O, and S M de Almeida-Santos. 2013. Composition, Species Richness and Abundance of Anurans in a Cerrado and Atlantic Forest Remnant of the São Paulo State, Brazil. Biota Neotrop. 13(1): 265–75.

Arruda, M P et al. 2011. Contemporary Gene Flow and Weak Genetic Structuring in Rococo Toad (*Rhinella Schneideri*) Populations in Habitats Fragmented by Agricultural Activities. Amphib. Reptil. 32(3): 399–411.

Avise, J. C. (1993); Molecular Markers, Natural History and Evolution. Chapman and Hall. ISBN: 0-412-03771-8(hb). ISBN: 0-412-03981(pb). *Journal of Evolutionary Biology* 7(6): 766–67.

Ayres, M., Ayres, Jr. M., Ayres, D.L., Santos, A.A.S. 2007. BioEstat- Statistical applications in the areas of biomedical sciences. Belém/PA: Sociedade Civil Mamirauá.

Becker, C G, C R Fonseca, C F B Haddad, and P I Prado. 2010. Habitat Split as a Cause of Local Population Declines of Amphibians with Aquatic Larvae: Contributed Paper. *Conserv. Biol.* 24(1): 287–94.

Beebee, T J C, and R A Griffiths. 2005. The Amphibian Decline Crisis: A Watershed for Conservation Biology? 366 *Biol. Conserv.* 125(3): 271–85.

Bernarde, P S, and L C Macedo. 2008. The Impact of Deforestation and Pastures on the Leaf-Litter Frog Community in Rondônia, Brazil. *Iheringia - Sér. Zool.* 98(4): 454–59.

Berry, O. 2001. Genetic Evidence for Wide Dispersal by the Sand Frog, *Heleioporus Psammophilus* (Anura: Myobatrachidae), in Western Australia. *J. Herpetol.* 35(1): 136–41.

Blaustein, A R, D B Wake, and W P Sousa. 1994. Amphibian Declines: Judging Stability, Persistence, and Susceptibility of Populations to Local and Global Extinctions. *Conserv. Biol.* 8(1): 60–71.

Bohonak, A.J. 2002. IBD (isolation by distance): a program for analyses of isolation by distance. *J. Hered.* 93: 374 153–154. 375 Bonnet, E, and Y Van De Peer. 2002. Zt: A Software Tool for Simple and Partial Mantel Tests. *J. Stat. Sof* : 1–376 12.

Calleffo, M.E.V. 2002. Anfíbios in Técnicas de coleta e preparação de vertebrados para fins científicos e didáticos. Instituto Pau Brasil de História Natural, São Paulo. 43-74p.

Cortez, C.C., Suárez-Mayorga, A.M., López-López, F.J. 2006. Preparación y preservación de material científico in Técnicas de inventario y monitoreo para anfíbios de la región tropical. Conserv. Int. Colomb., 173-218.

Crawford, J A, and R D Semlitsch. 2007. Estimation of Core Terrestrial Habitat for
Stream-Breeding Salamanders and Delineation of Riparian Buffers for Protection of Biodiversity. *Conserv. Biol.* 21(1): 152–58.

D’Anunciação, P E R et al. 2013. Forest Fragments Surrounded by Sugar Cane Are More Inhospitable to Terrestrial Amphibian Abundance than Fragments Surrounded by Pasture. *Int. J. Ecol.* 8p.

Dixo, M, J P Metzger, J S Morgante, and K R Zamudio. 2009. Habitat Fragmentation Reduces Genetic Diversity and Connectivity among Toad Populations in the Brazilian Atlantic Coastal Forest. *Biol. Conserv.* 142(8): 1560–387 69.

Drummond, G.M., Martins, C.S., Machado, A.B.M., Sebaio, F.A., Antonini, Y. 2005. Biodiversidade em Minas Gerais: um atlas para sua conservação. Fundação Biodiversitas, Belo Horizonte, Brazil.

Duellman, W.E., Trueb, L. 1986. Biology of amphibians. McGraw-Hill, New York, USA.549-553p.

Estoup, A et al. 2010. Combining Genetic, Historical and Geographical Data to Reconstruct the Dynamics of Bioinvasions: Application to the Cane Toad Bufo Marinus. *Mol. Ecol. Resour.* 10(5): 886–901.

Ewers, R M, and R K Didham. 2006. Confounding Factors in the Detection of Species Responses to Habitat Fragmentation. *Biol. Rev. Camb. Philos Soc.* 81(1): 117–42.

Excoffier, L, G Laval, and S Schneider. 2005. Arlequin (Version 3.0): An Integrated Software Package for nPopulation Genetics Data Analysis. *Evol. Bioinform Online* 1: 47–50.

Excoffier, L, P E Smouse, and J M Quattro. 1992. Analysis of Molecular Variance Inferred from Metric Distances among DNA Haplotypes: Application to Human Mitochondrial DNA Restriction Data. *Genetics* 399 131(2): 479–91.

Fahrig, L, and G Merriam. 1985. Habitat Patch Connectivity and Population Survival. *Ecol.* 66(6): 1762–68.

Figueroa, J, and A J Green. 2002. How Frequent Is External Transport of Seeds and Invertebrate Eggs by Waterbirds? A Study in Doana, SW Spain. *Archiv. Hydrobiologie* 155(4): 557–65.

Forman, R.T.T., Godron, M. 1986. Landscape Ecology. John Wiley & Sons, New York, USA.620p.

Frankham, R., Ballou, J.D., Briscoe, D.A. 2009. Introduction to conservation genetics, 2nd Edition. Cambridge University, Press,Cambridge, 618 p.

Gemmell, N J, V J Metcalf, and F W Allendorf. 2004. Mother’s Curse: The Effect of mtDNA on Individual Fitness and Population Viability. *Trends Ecol. Evol.* 19(5): 238–44.
Goebel, A M, J M Donnelly, and M E Atz. 1999. PCR Primers and Amplification Methods for 12S Ribosomal DNA, the Control Region, Cytochrome Oxidase I, and Cytochrome b of Bufonids and Other Frogs, and an Overview of PCR Primers Which Have Amplified DNA in Amphibians Successfully. *Mol. Phylogenet Evol.* 411(1): 163–99.

Gustafson, E J, and R H Gardner. 1996. The Effect of Landscape Heterogeneity on the Probability of Patch Colonization. *Ecology* 77(1): 94–107.

Hammer, Ø., Harper, D.A.T., Ryan, P.D. 2001. PAST, Paleontological statistics software package for education and data analysis Palaeontol Electronica.

Hanski, I, and M Gilpin. 1991. Metapopulation Dynamics: Brief History and Conceptual Domain. *Biol. J. Linn. Soc.* 417(1): 3–16.

Hitchings, S P, and T J C Beebee. 1998. Loss of Genetic Diversity and Fitness in Common Toad (Bufo Bufo) Populations Isolated by Inimical Habitat. *J. Evol. Biol.* 11(3): 269–83.

Hof, C, M B Araújo, W Jetz, and C Rahbek. 2011. Additive Threats from Pathogens, Climate and Land-Use Change for Global Amphibian Diversity. *Nature* 480(7378): 516–19.

Isaacs Cubides, P J, and J N Urbina Cardona. 2011. Anthropogenic Disturbance and Edge Effects on Anuran Assemblages Inhabiting Cloud Forest Fragments in Colombia. *Natureza & Conservação* 9(1): 39–46.

IUCN. An Analysis of Amphibians on the 2015 IUCN Red List: an online reference available at [http://cmsdocs.s3.amazonaws.com/summarystats/2015-4_Summary_Stats_Page/Documents/2015_4_RL_Stats_Table_4a.pdf](http://cmsdocs.s3.amazonaws.com/summarystats/2015-4_Summary_Stats_Page/Documents/2015_4_RL_Stats_Table_4a.pdf) on 3 May 2017.

Llewelyn, J et al. 2010. Locomotor Performance in an Invasive Species: Cane Toads from the Invasion Front Have Greater Endurance, but Not Speed, Compared to Conspecifics from a Long-Colonised Area. *Oecologia* 429 162(2): 343–48.

Manni, F, E Guérard, and E Heyer. 2004. Geographic Patterns of (Genetic, Morphologic, Linguistic) Variation: 431 How Barriers Can Be Detected by Using Monmonier’s Algorithm. *Human Biol.* 76(2): 173–90.

Moore, J A, D A Tallmon, J Nielsen, and S Pyare. 2011. Effects of the Landscape on Boreal Toad Gene Flow: Does the Pattern-Process Relationship Hold True across Distinct Landscapes at the Northern Range Margin? *Mol. Ecol.* 20(23): 4858–69.

Moritz, C. 1994. Defining ‘Evolutionary Significant Units’ for Conservation. *Trends Ecol. Evol.* 9(10): 373–75.

Moritz, C, T E Dowling, and W M Brown. 1987. Evolution of Animal Mitochondrial DNA: Relevance for Population Biology and Systematics. *Annu. Rev. Ecol. Syst.*. Vol. 18: 269–92.
Myers, N et al. 2000. Biodiversity Hotspots for Conservation Priorities. *Nature* 403(6772): 853–58.

Neckel-Oliveira, S. 2004. Effects of Landscape Change on Clutches of Phyllomedusa Tarsius, a Neotropical Treefrog. *Biol. Conserv.* 118(1): 109–16.

Neckel-Oliveira, S, and C Gascon. 2006. Abundance, Body Size and Movement Patterns of a Tropical Treefrog in Continuous and Fragmented Forests in the Brazilian Amazon. *Biol. Conserv.* 128(3): 308–15.

Pardini, R, S M De Souza, R Braga-Neto, and J P Metzger. 2005. The Role of Forest Structure, Fragment Size and Corridors in Maintaining Small Mammal Abundance and Diversity in an Atlantic Forest Landscape. *Biol. Conserv.* 124(2): 253–66.

Pechmann, J H K et al. 1991. Declining Amphibian Populations: The Problem of Separating Human Impacts from Natural Fluctuations. *Science* 253(5022): 892–95.

Pimm, S L, and P Raven. 2000. Biodiversity. Extinction by Numbers. *Nature* 403(6772): 843–45.

Pineda, E, and G Halffter. 2004. Species Diversity and Habitat Fragmentation: Frogs in a Tropical Montane Landscape in Mexico. *Biol. Conserv.* 117(5): 499–508.

Pittman, S E, M S Osbourn, and R D Semlitsch. 2014. Movement Ecology of Amphibians: A Missing Component for Understanding Population Declines. *Biol. Conserv.* 169: 44–53.

Pröhl, H, and A Krug. 2013. Population Genetics in a Fragmented Population of the European Tree Frog (Hyla Arborea). *Amphib-Reptil* 34(1): 95–107.

Renjifo, L M. 1999. Composition Changes in a Subandean Avifauna after Long-Term Forest Fragmentation. *Conservation Biology* 13(5): 1124–39.

Ribeiro-Júnior, J W, and J Bertolucisp. 2009. Anurans of the Cerrado of the Estação Ecológica and the Floresta Estadual de Assis, Southeastern Brazil. *Biot. Neotrop.* 9(1): 207–16.

Ricketts, T H. 2001. The Matrix Matters: Effective Isolation in Fragmented Landscapes. *Amer. Nat.* 158(1): 87–463 99.

Rogers, A R, and H Harpending. 1992. Population Growth Makes Waves in the
Distribution of Pairwise Genetic Differences. *Mol. Biol. Evol.* 9(3): 552–69.

Santos-Barrera, G, and J N Urbina-Cardona. 2011. The Role of the Matrix-Edge Dynamics of Amphibian Conservation in Tropical Montane Fragmented Landscapes. *Rev. Mex. Biodiv.* 82(2): 679–87.

Schabetsberger, R et al. 2004. Delineation of Terrestrial Reserves for Amphibians: Post-Breeding Migrations of Italian Crested Newts (Triturus C. Carnifex) at High Altitude. *Biol. Conserv.* 117(1): 95–104.

Semlitsch, R.D., Scott, D.E., Pechmann, J.H.K., Gibbons, J.W. 1996. Structure and dynamics of an amphibian community: evidence from a 16-year study of a natural pond. In: Long-term studies of vertebrate communities, p. 217-248. Cody, M.L., Smallwood, J.A., Eds, Academic Press Inc. San Diego.

Silva, R A, I A Martins, and D C Rossa-Feres. 2011. Environmental Heterogeneity: Anuran Diversity in nHomogeneous Environments. *Zoo.* 28(5): 610–18.

Sinsch, U et al. 2012. Connectivity of Local Amphibian Populations: Modelling the Migratory Capacity of Radio-Tracked Natterjack Toads. *An. Conserv.* 15(4): 388–96.

Smith, M A, and D M Green. 2005. Dispersal and the Metapopulation Paradigm in Amphibian Ecology and Conservation: Are All Amphibian Populations Metapopulations? *Ecog.* 28(1): 110–28.

Stuart, S N et al. 2004. Status and Trends of Amphibian Declines and Extinctions Worldwide. *Science* 480 306(5702): 1783–86.

Sturaro, M J, and V X da Silva. 2010. Natural History of the Lizard Enyalius Perditus (Squamata: Leiosauridae) from an Atlantic Forest Remnant in Southeastern Brazil. *J. Nat. Hist.* 44(19–20): 1225–38.

Tamura, K et al. 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* 28(10): 2731–39.

Urbina-Cardona, J N, M Olivares-Pérez, and V H Reynoso. 2006. Herpetofauna Diversity and Microenvironment Correlates across a Pasture-Edge-Interior Ecotone in Tropical Rainforest Fragments in the Los Tuxtlas Biosphere Reserve of Veracruz, Mexico. *Biol. Conserv.* 132(1): 61–75.

Wassens, S, R J Watts, A Jansen, and D Roshier. 2008. Movement Patterns of Southern Bell Frogs (Litoria Raniformis) in Response to Flooding. *Wildlife Research* 35(1): 50–58. Watson, D M. 2002. A Conceptual Framework for Studying Species Composition in Fragments, Islands and Other Patchy Ecosystems. *J. Biog.* 29(5–6): 823–34.

Watson, D.M. 2003. Long-Term Consequences of Habitat Fragmentation - Highland Birds in Oaxaca, Mexico. 493 *Biol. Conserv.* 111(3): 283–303.
Wiens, J.A. 1994. Habitat fragmentation, island v landscape perspectives on bird conservation. Ibis 137: 97-495 104.
Wilbur, H M. 1987. Regulation of Structure in Complex Systems: Experimental Temporary Pond Communities. Ecol. 68(5): 1437–52.
Wright, S. 1931. Evolution in mendelian populations. Genetics 16: 97-159.

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     | 014.24 | 026.35 | 020.88 | 017.84 | 009.06 | 020.11 | 088.71 |
| P3     | 029.93 | 026.73 | 023.75 | 018.30 | 009.38 | 075.99 |
| S1     | 006.39 | 008.80 | 035.13 | 025.84 | 102.92 |
| S2     | 003.68 | 029.21 | 025.00 | 101.79 |
| S3     | 006.39 | 008.80 | 035.13 | 025.84 | 102.92 |
| C1     | 023.57 | 020.11 | 018.30 | 009.38 | 075.99 |
| C2     | 025.82 | 021.60 | 098.65 |
| PNB    | 025.85 | 087.26 |

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).

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  | Fst=0.1482 | 0.1183   | 14.82 |
| PNB      | Fst=0.1644 | 0       | 14.01 |
| Matrices | Fst=0.2882 | 0       | 71.17 |
| 2groups  | Fst=0.2  | 0.2541   | 12    |
| PNB      | Fst=0.1844 | 0.0039  | 16.23 |

| Pasture | Fst=0.2823 | 0 | 71.77 |
|---------|------------|---|------|
| 2groups | Fst=-0.031 | 0.5161 | -3.09 |
| PNB     | Fst=0.1593 | 0.1007 | 16.42 |
| Sugar   | Fst=0.1332 | 0.0283 | 86.68 |

| Pasture | Fst=0.3901 | 0.3314 | 39.02 |
|---------|------------|--------|------|
| 2groups | Fst=-0.016 | 0.5914 | -1.59 |
| PNB     | Fst=0.1629 | 0.001  | 16.55 |
| Sugar   | Fst=0.1496 | 0.001  | 85.04 |

| Pasture | Fst=-0.020 | 0.8025 | -2.03 |
|---------|------------|--------|------|
| 2groups | Fst=0.1477 | 0.0029 | 15.07 |
| Sugar   | Fst=0.1363 | 0      | 86.97 |

| Sugar   | Fst=0.1536 | 0.2258 | 15.36 |
|---------|------------|--------|------|
| Coffee  | Fst=0.1094 | 0.1124 | 9.26  |

| Coffee  | Fst=0.2462 | 0.001  | 75.38 |
|---------|------------|--------|------|
| 4groups | Fst=0.1808 | 0.1808 | 8.51  |
| PNB     | Fst=0.1501 | 0.0029 | 15.73 |
| Pasture | Fst=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 0.167 | P1 0.067 | P2 0.133 | P3 0.467 | S1 0.333 | S2 0.125 | S3 0.333 | C1 0.133 | C2 0.500 |
|-----------|-----------|----------|----------|----------|----------|----------|----------|----------|----------|
| H1        |           |          |          |          |          |          |          |          |          |
| 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    | 0.200    | 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        | 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        | 20       | 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
Moreno-Cotulio.sqn CT290 KF974581
Moreno-Cotulio.sqn CT291 KF974582
Moreno-Cotulio.sqn CT292 KF974583
Moreno-Cotulio.sqn CT293 KF974584
Moreno-Cotulio.sqn CT294 KF974585
Moreno-Cotulio.sqn CT295 KF974586
Moreno-Cotulio.sqn CT297 KF974587
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
Moreno-Cotulio.sqn CT292 KF974583
Moreno-Cotulio.sqn CT293 KF974584
Moreno-Cotulio.sqn CT294 KF974585
Moreno-Cotulio.sqn CT295 KF974586
Moreno-Cotulio.sqn CT297 KF974587
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
| Fragment ID     | Location 1                                    | Location 2                                    |
|----------------|-----------------------------------------------|-----------------------------------------------|
| 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              |
CT1139 fragment of sugar cane matrix (S2)  21° 25’ 26.02” S 46° 7’ 30.43” W
CT1140 fragment of sugar cane matrix (S2)  21° 25’ 26.02” S 46° 7’ 30.43” W
CT2184 fragment of sugar cane matrix (S2)  21° 25’ 26.02” S 46° 7’ 30.43” W
CT2186 fragment of sugar cane matrix (S2)  21° 25’ 26.02” S 46° 7’ 30.43” W
CT2187 fragment of sugar cane matrix (S2)  21° 25’ 26.02” S 46° 7’ 30.43” W
CT380 fragment of sugar cane matrix (S2)  21° 25’ 26.02” S 46° 7’ 30.43” W
CT1141 fragment of sugar cane matrix (S2)  21° 25’ 26.02” S 46° 7’ 30.43” W
CT1142 fragment of sugar cane matrix (S2)  21° 25’ 26.02” S 46° 7’ 30.43” W
CT1143 fragment of sugar cane matrix (S3)  21° 25’ 31.21” S 46° 5’ 35.77” W
CT1144 fragment of sugar cane matrix (S3)  21° 25’ 31.21” S 46° 5’ 35.77” W
CT1145 fragment of sugar cane matrix (S3)  21° 25’ 31.21” S 46° 5’ 35.77” W
CT1146 fragment of sugar cane matrix (S3)  21° 25’ 31.21” S 46°
CT1138 fragment in coffee matrix (C2)   21° 33’ 47.04” S 45° 56’ 14.74” W

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