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Genetic analysis of tarantulas in the genus *Brachypelma* using Inter Simple Sequence Repeats (ISSR)

By

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Abstract

There is a great deal of morphological and genetic species diversity on Earth that requires careful conservation. One such genetically diverse genus of tarantulas is that of *Brachypelma*. In this study, we employ a newer DNA fingerprinting technique known as Inter Simple Sequence Repeat (ISSR), to study the genetic variation among *Brachypelma* species and to determine if the invasive *Brachypelma* tarantula found in Florida *B. vagans.* Although *B. vagans* is a species protected under CITES Appendix II, this species has a wide distribution in Mexico and traits allowing for invasion to new habitats. It was hypothesized that the invasive tarantula in Florida is that of *B. vagans* and that it would be more closely related to samples from the Mexican populations as opposed to samples from the United States pet trade. DNA results showed that the specimen in Florida is in fact *B. vagans*, however, it is more closely related to its Mexican relatives. The ability of species to invade other non-endogenous habitats is an interesting phenomenon. It is not known how this species invasion of *B. vagans* will affect the natural Florida habitat and its native species.

Introduction

There is a vast biodiversity on Earth in both morphological traits and molecular makeup. The conservation of biodiversity is a key challenge in the 21st century. More species are classified as endangered each year, and many of these species are in dire circumstances due to inbreeding (Hedrick & Kalinowski, 2000). Inbreeding can occur through geographical isolation of populations (da Silva et al., 2016). The pet trade can also result in inbreeding. Inbreeding can cause a once genetically diverse species to decline in numbers and genetic diversity leading to endangerment and even possibly extinction. The amount of genetic diversity prior to such

inbreeding events can vary among different species of the same genus, but, in general, species of the same genus are relatively closely related with only 10-100 million nucleotide differences in protein-coding DNA sequences (Wink, 2006). Many factors can affect the relative molecular relatedness of species within a genus including range of distribution, dispersal capacity of the organisms, and exposure to teratogens, chemical or environmental toxins that could lead to embryo malformation (Lüddecke et al., 2018). These molecular differences can sometimes, but not always, result in morphological differences in organisms that are easily observable to the naked eye.

Morphological and genetic differences between animal species are commonly shown via using phylogenetic trees. Phylogenies show the relative evolutionary relationship between different species and how closely related they are in evolutionary history. Phylogenies can be created using either morphological or genetic data. Many preliminary phylogenies of organisms are made using easily observable, morphological differences (Wiens, 1999). Phylogenies that are based on morphological differences require genetic testing to show genomic similarity that results in the morphological similarities in order to verify the morphological phylogenies (Livezey & Zusi, 2007). Phylogenies that are supported with genetic data on top of the already present morphological data are more reliable and evolutionarily accurate (Wiens, 1999). Not only is there diversity between different species on a phylogenetic tree, but there is also diversity within certain clades and species on these trees.

One very diverse group of organisms are tarantulas, however, their phylogenies are not well developed and are lacking in molecular data. Rudimentary phylogenies have been created for this group, however, they are largely based on morphological data and analysis (Foley et al.,

2018). There are many different subfamilies and genera belonging to each subfamily within the family *Theraphosidae,* the family housing tarantulas. One such subfamily is Theraphosinae which houses the *Brachypelma* genus of tarantulas (Mendoza & Francke, 2017). Due to few morphological differences in the species of the *Brachypelma* genus, the substantiation of these rudimentary phylogenies requires more molecular data (Lüddecke et al., 2018). *Brachypelma* is comprised of at least 20 species divided into two clades (known as complexes): Emilia and Vagans (Petersen et al., 2007). Molecular data have shown the genetic relationship between different species in the Emilia complex, however, this type of data has not been collected for the entire genus (Mendoza & Francke, 2017).

The two *Brachypelma* complexes (Emilia and Vagans) are both expected to be monophyletic based on analysis of the mitochondrial DNA of this genus of tarantulas, however, nuclear DNA has not been completely explored (Mendoza & Francke, 2017). Inter-species geneflow could affect the monophyletic distribution of the species in the Emilia and Vagans complexes. For example, this could occur when males travel between different populations of the same species and reproduce with females of a separate population (Longhorn et al., 2007). Not only does gene flow between different species affect the genomic makeup of organisms, so does isolation from inadvertently introducing an organism to a new region.

Tarantulas of the *Brachypelma* genus are considered endangered and are protected under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) appendix II (Longhorn et al., 2007). This genus consists of a vibrantly colored group of tarantulas known for their distinct red hair and thus have become quite popular in both international and United States pet trade (Turner et al., 2018). Other factors that contribute to

these tarantulas belonging to the CITES protected list include habitat destruction in their native areas and a high juvenile mortality rate and late sexual maturity (Machkour-M'Rabet et al., 2009).

Brachypelma tarantulas are native to the Caribbean and Pacific coast of Mexico, however, they have been found outside of these regions in recent years despite being a protected genus under CITES (Fig. 1). Although native to coastal regions of Mexico, approximately 40-50 years ago, individuals of *Brachypelma vagans*, the Red Rump tarantula, were found in Florida (Edwards & Hibbard, 1999.; Machkour-M'Rabet et al., 2012). It is possible that this invasion is due to the pet trade, because *B. vagans* is a very popular tarantula species in the United States pet trade (Turner et al., 2018). The lack of other *Brachypelma* species, including other *B. vagans* populations in Florida makes this population interesting to compare to other populations of *B. vagans* that have intra-species gene flow between populations. It is expected that the invasive Florida population of *B. vagans* has experienced less gene flow due to geographical isolation from other species of *B. vagans*.

Figure 1. Distribution of *Brachypelma* tarantulas in Mexico (Locht et al., 1999)

There is a wide variety of methods used to study the genetic and molecular relatedness of different organisms. One common approach is DNA fingerprinting, which involves PCR of isolated DNA samples and analysis of gel electrophoresis patterns (Sucher et al., 2012). DNA fingerprinting methods are very suitable to many studies because of their relatively low cost, large number of DNA markers that can be studied at once, small amount of initial DNA required, and relatively quick time frame to amplify and analyze markers (Sobotka et al., 2004). Such methods include AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), SSR polymorphism (simple sequence repeats), and ISSR (inter simple sequence repeats) (Nybom, 2004). AFLP uses two common restriction enzymes to digest DNA, MseI and PstI, create sticky ends, and then PCR primers that match these restriction enzyme cut sites are used to amplify the DNA (Wink, 2006). AFLP produces consistent results, however, it is quite costly (Blair et al., 1999). RADP has long been one of the more popular methods for fingerprinting DNA because it is simple and fast method and does not require any prior knowledge of the DNA sequence in question; however, it has low reproducibility of results (Archak et al., 2003). Microsatellites are short tandem repeats in the genetic sequence and have long been used in SSR polymorphism methods for genetic studies because of the ability to easily create PCR primers and get efficient results, however, species-specific primers need to be made and this can be time consuming and lead to difficulties (Blair et al., 1999). Each method has both advantages and disadvantages to its use, however, ISSR gives similar results as AFLP with fewer, simpler steps (Wink, 2006). ISSR also shows higher reproducibility than RAPD methods (Archak et al., 2003). Lastly, ISSR primers are universal and not species-specific as are its counterparts for traditional SSR polymorphism methods (Blair et al., 1999).

There were two primary goals of this research project. The first goal was to study how species of the *Brachypelma* genus are related and to understand how organisms from different populations of the same species are related using ISSR-PCR techniques. ISSR was used to study the genetic relationship between different species of the *Brachypelma* genus and various populations belonging to *Brachypelma vagans*. Samples were collected from Mexico, Florida, the United States pet trade, and from Nicaragua. The samples from Nicaragua have been placed in the *Brachypelma* genus, however, further analysis needs to be done to place the samples into a species, or at least complex (Emilia or Vagans). The second goal of this research project was to determine if the invasive *Brachypelma* specimen in Florida are in fact *B. vagans,* and if they are more closely related to Mexican indigenous specimen or U.S. pet trade samples. We predict that *B. vagans* collected in Florida would be more closely related to the Mexico *B. vagans* due to recent introduction into the Florida habitat compared to the pet trade specimen who have been inbred for decades.

Materials and Methods:

Brachypelma Sample Acquisition

To study the genetic relationship among tarantula species in *Brachypelma* and to determine if tarantulas discovered in Florida belong to the taxon *B. vagans,* we used Inter Simple Sequence Repeats (ISSR) (Machkour-M'Rabet et al., 2009). Tarantulas from the genus *Brachypelma* were collected from various locations in Mexico, the United States pet trade via the tarantula breeder Netbug, the invasive population in Florida, and a population of unknown species in Nicaragua (Table 1). Each individual tarantula was considered a sample and these samples were collected in the or were ordered online using the United States pet trade system.

Table 1. Tarantula sample data table.

Total Sample Numbers	Tarantula Species	Complex	U.S. Pet Trade	Florida	Mexico
22	B. Vagans	Vagans	5	1	16
1	B. Albopilosum	Vagans	1	-	-
2	B. Smithi	Emilia	2	-	-
1	B. Sabulosum	Vagans	1	-	-
1	B. Baumgarteni	Emilia	1	-	-
4	B. Boehmei	Emilia	4	-	-
5	B. Epicurianum	Vagans	-	-	5
2	B. Emlia	Emilia	2	-	-
3	Brachypelma (Nicaragua)	Unknown	3	-	-
4	Crassicus Lamanai	N/A	-	-	4
3	Grammostola Rosae	N/A	3	-	-

DNA Extraction

We extracted DNA from tarantula samples with a lysis buffer protocol used in previous studies on *Brachypelma* so we could study the molecular inter simple sequence repeat (ISSR) markers of these organisms (Table 1) (Machkour-M'Rabet et al., 2009). An entire tarantula was used for small individuals and the third leg was used for large individuals. The abdomen, however, was excluded from extraction in order to prevent the DNA of ingested organisms

from interfering with the study. Tissues were incubated at 50°C overnight in 350 μL lysis buffer (Tris-HCl, EDTA, NaCl solution, and ultra-pure water), 40 μL 10% SDS solution, and 25 μL Proteinase K. Next, 200 μL 6M NaCl solution was added into the Eppendorf tubes and centrifuged for 30 minutes at 13,000 rpm at 4°C. The supernatant was transferred into new Eppendorf tubes, 400 μL cold isopropanol added, and the samples then centrifuged again for 40 minutes at 13,000 rpm at 4°C. The isopropanol was eliminated and the pellets retained. 500 μL cold ethanol (70%) was added to the pellet, and finally, the samples were centrifuged for 10 minutes at 13,000 rpm at 4°C. The ethanol was then eliminated, the pellets allowed to dry, and 50 μL ultra-pure water was added to the Eppendorf tubes to resuspend the DNA, and was incubated for 1 hour at 42°C. The extracted DNA tubes then were put directly into the freezer (-20°C) until amplification.

An initial gel electrophoresis was performed on a 1% agarose gel in X1 TBE solution using the extracted DNA to test for the presence and quality of the DNA. Once the gel electrophoresis was completed and the quality of DNA confirmed, quantification was performed with a Qubit fluorimeter machine (Invitrogen**)** to determine how much DNA was present in the samples. Sufficient DNA for further testing requires approximately 30 ng/uL. If samples contained more than 30 ng/uL, dilutions were performed using ultra-pure water. Samples with inadequate DNA quantity and/or quality were discarded from the experiment before proceeding to the PCR process.

Initial PCR Amplification

The DNA was amplified with PCR techniques using five different primer sets: +$(GACA)_4$, $(GAG)_5GC$, $(GACA)_4$+, $(AG)_8G$, and $(GTG)_5GC$. (Table 2). These primers are common ISSR primers

and have been used previously with tarantulas of the *Brachypelma* genus (Machkour-M'Rabet et al., 2009). Amplifications were completed in a 15 μL solution with 0.3 μL primer, 1 μL DNA, 1.5 μL x5 Green Buffer (Promega), 0.3 μL dNTP mix (Promega), 1.8 μL $MgCl_2$, 0.25 μL GoTaq Flexi DNA Polymerase (Promega), and 10.85 μL ultra-pure H_2O. The protocol for the PCR program that was used includes initial denaturation at 94°C for 4 minutes, 39 cycles of denaturation at 94°C for 45 s, annealing at X°C (X= +$(GACA)_4$ at 60°C, $(GAG)^5GC$ at 63°C, $(GACA)_4$+ at 57°C, $(AG)_8G$ at 56°C, and $(GTG)_5GC$ at 66°C) for 45 s, and extension at 72°C for 2 min, and a final extension step at 72°C for 10 minutes. These amplified samples were run on a 2% agarose gel in 1X TBE solution at 100 V for approximately 2 hours to allow thorough

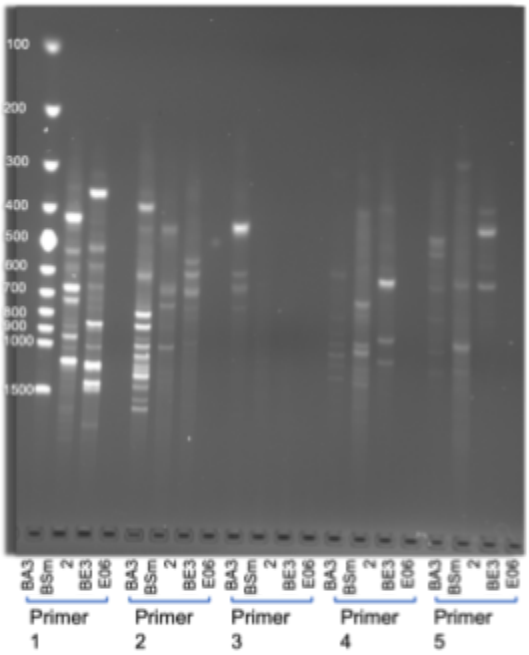


Figure 2. Example of gel electrophoresis for ISSR obtained from initial PCR Revised PCR Amplification

separation of different sized ISSR bands. All gel images were taken using a Bio-Rad molecular imaging system (see example of ISSR gel in Fig. 2).

We completed the PCR amplification in a 25 μL solution with 1 μL primer, 2 μL DNA (~40 ng), 0.5 μL dNTP mix (Promega), 2.5 μL x5 Green Buffer (promega), 3 μL $MgCl_2$, 0.5 μL GoTaq Flexi DNA Polymerase (Promega), and 15.5 μL ultra-pure H_2O. The PCR protocol was run for 48 samples using a total of 4 different primers. All amplifications were run on a 2% Agarose gel in 1X TBE solution at 100 V for approximately 2 hours. The resulting gel images were used for analysis of ISSR (Inter Simple Sequence Repeat) bands of the different *Brachypelma* samples to create a binary matrix database.

Table 2. –ISSR Primers Used in Study. B = T, C, or G. W = A or T.

Code	Sequence	Abbreviation	Ultimately Used?
1	WBGACAGACAGACAGACA	$+(GACA)_4$	Yes
2	GAGGAGGAGGAGGAGGC	$(GAG)_5GC$	Yes
3	GACAGACAGACAGACAWB	$(GACA)_4+$	No
4	AGAGAGAGAGAGAGAGG	$(AG)_8G$	Yes
5	GTGGTGGTGGTGGTGGC	$(GTG)_5GC$	Yes

Genetic Distance Determination

Gels were scored in order to create a database that shows a mathematical representation of genetic similarities. This involved marking a band of a certain size for all samples and designating with a numeric code. This process was repeated until all bands had been marked on the gel with a code. This information was then entered into an Excel database using a binary matrix protocol of band presence/absence. This was done for every sample for every primer (a total of 4 gels).

The binary matrix data was analyzed using two programs: GenAlEx and MEGA. GenAlEx, an Excel add-on program, directly used the data set created by scoring the ISSR bands for each primer to make a tri-matrix database. A tri-matrix database is a statistical tool that has a goal of learning associations between two different factors which in this case are geographical location and genetic relatedness (Park & Hwang, 2017). We then performed a principle coordinates analysis (PCoA) on the tri-matrix data set to determine relative genetic distance between the different samples. This procedure was done multiple times for different combinations of samples. It was done for *Brachypelma vagans* only, *Brachypelma* species only, and for all of the samples.

For the PCoA of all the samples (all *Brachypelma*, *Grammastola*, and *Crassicus*), there appeared to be some outliers (Fig. 3). The unlikeliness of these outliers was determined by whether one individual was in a vastly different location than the rest of the members in that species or if one specimen was in a different location than would be expected for the complex to which that species belongs. Two *B. vagans* from the Mexico samples were located far from the rest of the Mexican *B. vagans* and closer to samples of the Emilia complex. These outliers were removed from the study as we were unsure if they were truly accurate outliers or if some

other human error had led to these results (ex. mislabeling, contamination, etc). Once the outliers were removed, a subsequent PCoA analysis was completed using only samples of the *Brachypelma* genus.

Phylogenetic Relationships

Once the PCoA analysis was complete, the MEGA program was then used to create phylogenetic trees (UPGMA and Neighbor-Joining) based off of the molecular evidence we collected with the ISSR protocol. Both UPGMA and Neighbor-Joining trees start out their trees by grouping the two taxa with the smallest distance and progressively adding more distant taxa to the group or groups. The difference is that UPGMA assumes the data to be ultrametric while Neighbor-Joining relaxes this assumption and assumes instead that the data are additive (Morrison, 1996). In total, four trees were created: 1) UPGMA – *Brachypelma vagans* only, 2) Neighbor-Joining – *Brachypelma vagans* only, 3) UPGMA – all *Brachypelma,* and 4) Neighbor-Joining – all *Brachypelma.*

Results

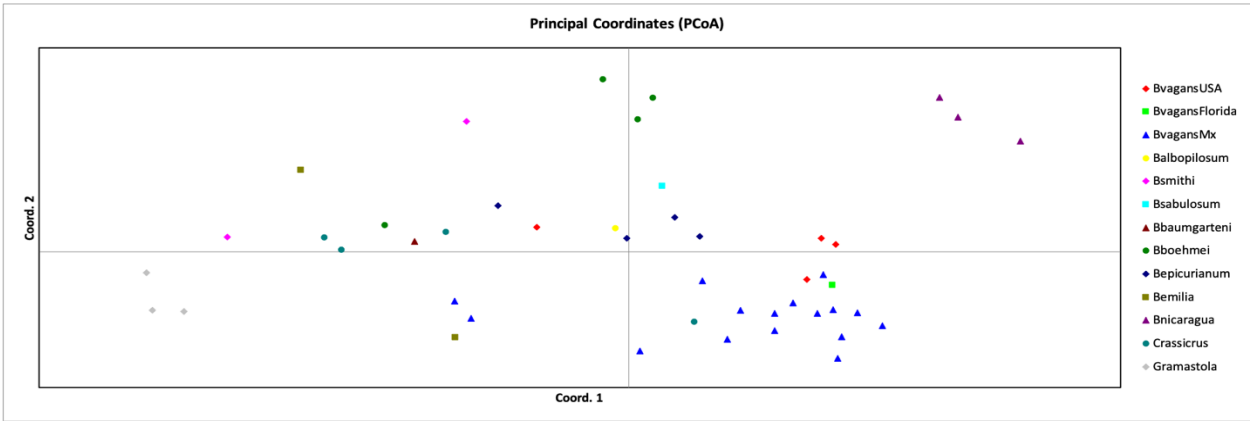
Genetic Distance

The binary matrix database created by scoring the ISSR gels was used with the GenAlEx program to determine genetic relatedness using a PCoA analysis. This was done for *Brachypelma vagans* samples alone as well as with all the different *Brachypelma* species resulting in two separate PCoA charts.

The initial PCoA for all the samples showed two outliers based off of species and relative location (Fig. 3). These outliers were two *B. vagans* samples outside of the range of the *Brachypelma* complex and instead were grouped with other species of the Emilia complex.

Other than these outliers, species were grouped within their respective complexes and species that were not of the *Brachypelma* genus were grouped relatively distantly from either of the complex groupings. These two species included *Crassicus Lamanai* and *Grammastola Rosae.*

Figure 3. Initial PCoA Analysis with All Samples. Initial PCoA shows good grouping of *B. vagans* minus some inconsistent outliers (circled in red).



A subsequent PCoA without the outliers was completed with only *Brachypelma* samples (Fig. 4). On this PCoA analysis, we were able to highlight a general area on the graph showing the distribution of species by their assigned complex: Emilia or Vagans (Fig. 4). Based on their positioning in the figure and the data presented, the three unknown *Brachypelma* samples from Nicaragua appear to belong within the genetic range of the Vagans complex as opposed to the Emilia complex.

On this PCoA analysis, there is evidence supporting a closer genetic relatedness of the *B. vagans* sample from Florida to those samples collected from the Mexican wild as opposed to those samples collected from the United States pet trade (Fig. 5). This is shown by the Florida sample being placed in closer proximity to the Mexican samples as opposed to the pet trade samples.

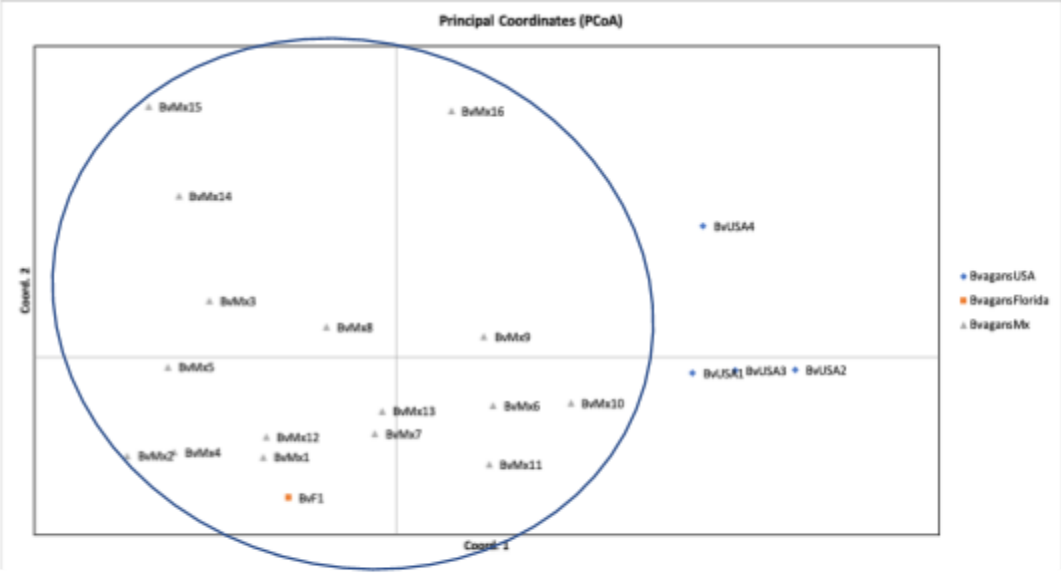


Figure 5. PCoA output showing distribution of different *B. vagans* samples: those from Mexico, U.S. pet trade, and Florida.

Phylogenetic Relationships

For the *Brachypelma vagans* only data, the trees (both UPGMA and Neighbor-Joining) show all the samples of *B. vagans* from the United States pet trade as producing a monophyletic group. These trees also both show the *B. vagans* sample from Florida as being more closely related to samples from Mexico. This coincides with the data that resulted from our PCoA analysis using GenAlEx.

Both the UPGMA and Neighbor-Joining trees of the *Brachypelma vagans* only data show the United States pet trade samples to be monophyletic. This is a significant finding showing closer genetic relatedness between the pet trade samples than the other samples involved.

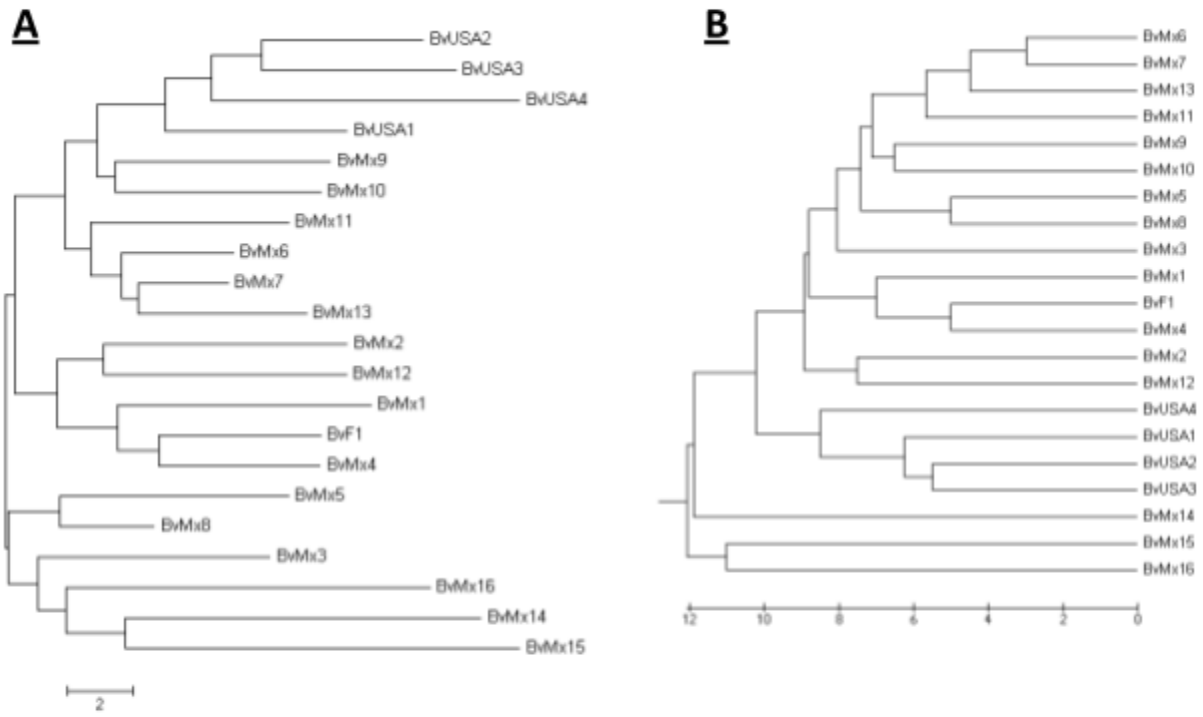


Figure 6: *Brachypelma Vagans* Neighbor-Joining Tree (A) and *Brachypelma Vagans* UPGMA Tree (B)**.** The distances shown indicate that *B. vagans* from Florida is closer related to Mexican samples genetically. The United States pet trade samples also are monophyletic in both trees.

In the phylogenetic trees made containing all *Brachypelma* samples, both the Neighbor-Joining and UPGMA trees have the three Nicaraguan samples in a monophyletic group. Also, based on the phylogenetic trees along with the data shown by the PCoA analysis, the Nicaraguan samples are more closely related to those samples of the Vagans complex and thus are likely a species of the Vagans complex as opposed to the Emilia complex.

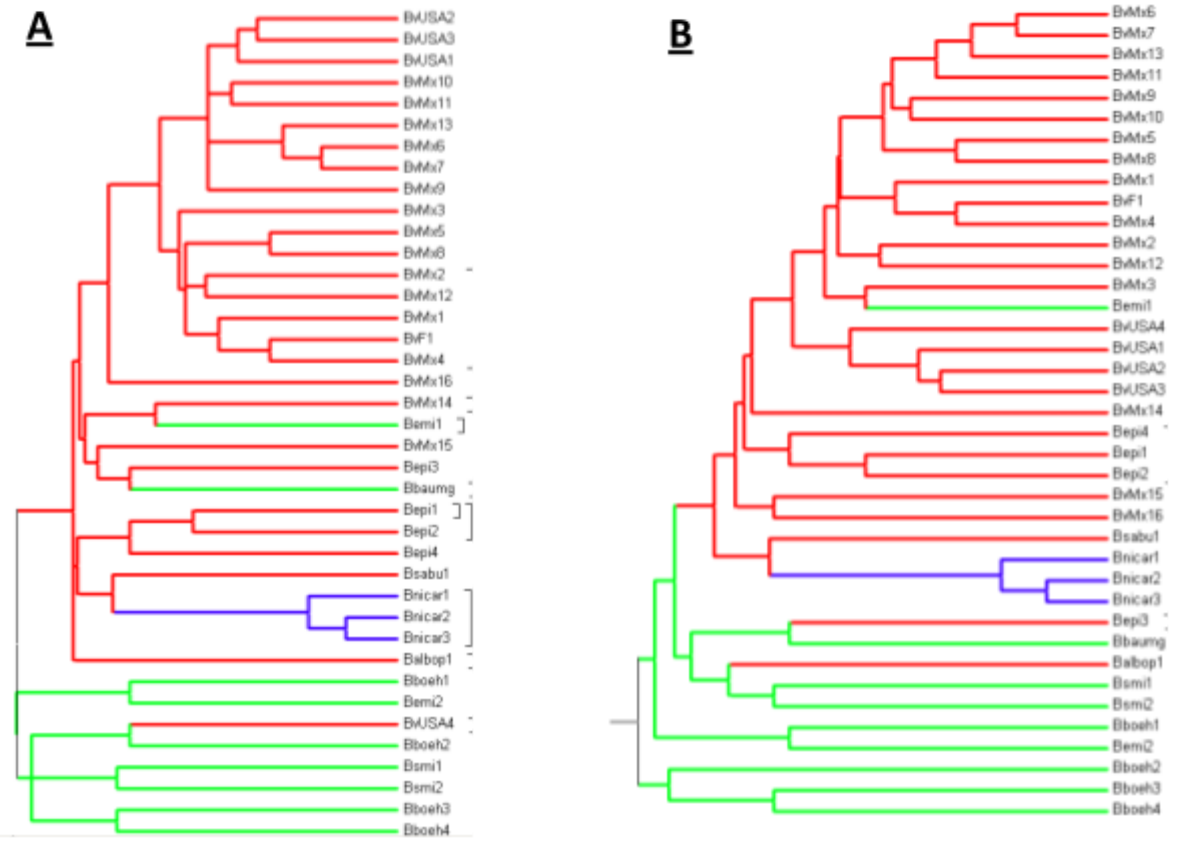


Figure 7: All *Brachypelma* Species Neighbor-Joining Tree (A) ad All *Brachypelma* Species UPGMA Tree. The distances shown indicate that Nicaraguan samples are closer related to other species of the Vagans complex. The Nicaraguan samples also are monophyletic in both trees.

Discussion

The two main goals to this study were to use Inter Simple Sequence Repeats (ISSR) to study how different species of *Brachypelma* organisms are related and to confirm findings based on morphological data that the invasive *Brachypelma* species in Florida is *B. vagans*. We were also interested in determining if this *B. vagans* population in Florida was closer related to United States pet trade samples or Mexican population samples. Related to the first goal, we

found that the unknown *Brachypelma* species from Nicaragua belonged to the Vagans complex as opposed to the Emilia complex. The findings related to the second goal indicated that the invasive species in Florida is actually *B. vagans* and that it is more closely related to Mexican samples than pet trade samples.

Our findings revealed clear evidence that the Florida *Brachypelma* samples belong to the species *B. vagans.* This supports the previous evidence based on morphogenetic markers that this invasive tarantula in Florida is, in fact, *Brachypelma vagans* (Edwards & Hibbard, 1999). This confirmation indicates that this species has successfully invaded the Florida area; the native range of *B. vagans* is southern Mexico south to Costa Rica (Machkour-M'Rabet et al., 2012). Of the *B. vagans* populations tested, the Florida population is more closely related to the Mexican samples than the samples obtained from the United States pet trade. This leaves us with future research to determine how Mexican specimen made it to Florida, as previous hypotheses attribute the presence of wild *B. vagans* in Florida to a single female having escaped from the pet trade (Edwards & Hibbard, 1999).

The presence of *B. vagans* in Florida is troublesome for two reasons: we do not know how they got there and we do not know what the effects of this invasive species will be on the Florida environment. In recent years, *B. vagans* has also successfully invaded Cozumel Island in Mexico (Machkour-M'Rabet et al., 2017). It is well documented that invasive species generally show less genetic diversity which is very disadvantageous for success of a species (Machkour-M'Rabet et al., 2017). Less genetic diversity of an invasive or separated population can lead to such phenomenon as the Founder Effect or bottleneck effect (Fukushima et al., 2019). It also should not be discounted that *B. vagans* is an organism protected by CITES for

endangerment and thus the invasion of this species could lead to tragedy for this tarantula. Protection under CITES appendix II occurs due to habitat destruction as well as illegal collection of the spiders for the pet trade (Turner et al., 2018).

The ability of this tarantula to successfully invade non-native localities leads researchers to question whether or not this tarantula needs to be on the CITES protection list and also the mechanisms used by this species to survive in a non-endogenous habitat. Some research has even shown that the Red Rump group of tarantulas (which includes *B. vagans*) should be downgraded on its conservation priority level based off of reproductive success (Turner et al., 2018). However, even though this tarantula has shown successful invasion in multiple areas, it is still dying off in some of its natural habitats (Machkour-M'Rabet et al., 2017).

This study also revealed evidence of the unknown Brachypelma samples from Nicaragua belonging to the Vagans complex as opposed to the Emilia complex of Brachypelma. These results were shown through the use of the GenAlEx program's PCoA analysis as well as MEGA's analysis and creation of phylogenetic trees (both UPGMA and Neighbor-Joining). These results are consistent with what has been known of the distribution of the Vagans complex as close relatives of these spiders are endogenous to Nicaragua (Turner et al., 2018). This conclusion alongside the evidence of the Florida population of *B. vagans* provides further evidence that the ISSR technique is useful in analyzing genetic relationships among tarantulas (Machkour-M'Rabet et al., 2009).

ISSR is an increasingly popular method of analyzing the genetic makeups of organisms and this is partially due to the structure and make up of these ISSR bands. There are four primary reasons that ISSR bands are becoming favored over other genomic techniques: 1) little

genomic DNA is required as starting material (less than 50 ng), 2) the ability to reproduce the results generated by ISSR techniques is high, 3) the overall cost for producing these bands is low, and 4) the procedure for producing these bands is simple (Ng & Tan, n.d.).

ISSR bands are portions of DNA surrounded on either side with identical, yet inverted, microsatellites (Blair et al., 1999). Microsatellites consist of short fragments of base pairs (e.g. CAG) that are repeated 10-20 times on average (Wink, 2006). Microsatellites flanking these portions of DNA allow for easy identification and creation of primers for PCRs (Machkour-M'Rabet et al., 2009). With ISSR, more closely related organisms have more commonalities in the positions and sizes of ISSR bands throughout their genome and ISSR band analysis can be used to construct phylogenies and determine genetic relationship between species (Wink, 2006).

ISSR has only been used once before in tarantulas (Machkour-M'Rabet et al., 2009), however, there are several advantages to using the newer ISSR technique for molecular and genetic analysis of tarantulas of the *Brachypelma* genus. The ISSR technique has been used relatively extensively to study many plant species and has shown good biochemical, morphological, and molecular marker agreement with species tested such as *Anacardium occidentale L.* (Archak et al., 2003). However, this technique has recently been used in other animals such as the Russian beef cattle and is deemed a good method based on being easy to use, easy to reproduce, and having a relatively low cost (Sulimova et al., 2016). One advantage of ISSR include that the existence of ISSR primers is universal among plant and animal species making the creation of primers for PCR simpler (Machkour-M'Rabet et al., 2009). Another advantage is that many segments of genomic DNA can be amplified with one primer as there

are multiple locations (up to 80 in various species) in a genome surrounded by these microsatellite portions to which the primers are complementary (Blair et al., 1999). A third advantage is that ISSR fragments are dominantly inherited (Ng & Tan, n.d.)

This is the second study to show good outcomes for ISSR bands of tarantulas of the genus *Brachypelma* (Machkour-M'Rabet et al., 2009). Until 2009, RAPD was the primary DNA fingerprinting method being used on tarantulas of the *Brachypelma* genus (Hettle et al., 1997). The ability of ISSR bands to be used on animals is a relatively new discovery. ISSR, however, has long been successfully used for the study of plant species, although, it is still less common than other DNA fingerprinting techniques such as RAPD and AFLP (Nybom, 2004). In recent past there have been comparative studies for RAPD, AFLP, and ISSR in plant species and one such study on *Anacardium occidentale L.* (cashew) showed ISSR as a feasible, more cost-efficient alternative to AFLP and RAPD (Archak et al., 2003).

While this study provided evidence that the ISSR marker technique of analysis is successful in studying relative relatedness of tarantulas of the *Brachypelma* genus, there are some limitations to our findings. Perhaps, the biggest limitation was lack of substantial samples of *B. vagans* from Florida to test. We only had one sample from Florida successful in the ISSR method and thus more research will need to be done in the future to study the genetic diversity of the Florida population. Studying the genetic diversity of the Florida population may give us more insight into how this protected species was able to successfully invade a new area outside of its endogenous habitat. Another limitation of this study was the relatively few samples we had for other species of the *Brachypelma* genus. We did not have samples for every species and those we did have samples for did not contain adequate sample numbers. Some species only

had one specimen. Future studies should include more individuals per species and should be conducted to further investigate the genetic diversity of the *Brachypelma* genus.

The results of this study are consistent with the only previous study of ISSR usage on tarantulas (Machkour-M'Rabet et al., 2009). ISSR is just as useful and possibly a better alternative than the use of other molecular marker techniques. Further research with other tarantula specimen of other genera should be conducted for further confirmatory evidence of the success and usefulness of ISSR markers for tarantulas. Research should also be done using other animals to determine the use of ISSR as a DNA fingerprinting technique in all of the animal kingdom as a preliminary, cheaper alternative to other methods.

This study and studies like it investigating the genetic diversity of endangered species are important to understanding the causes and consequences of loss of biodiversity (Hedrick & Kalinowski, 2000). Endangerment of more species has been happening due to human interference and it is vital that we investigate how to protect these precious, and increasingly rare creatures. As researchers we have the ability to understand the complex interactions that take place on our planet and use this information to protect all the plants and animals that habitat it.

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