Genetic Diversity of *Neckeropsis undulata* (Hedw.) Reich Populations in Old and Young Forest Stands

By

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ABSTRACT

Deforestation events are rapidly occurring in tropical countries; consequently forest fragments dominate the landscape. Fragmentation in Puerto Rico has shown to negatively affect bryophyte communities in forest remnants. Since bryophytes dispersal abilities are considered limited and fragmentation is expected to reduce genetic diversity of the isolated populations, the genetic diversity of populations of Neckeropsis *undulata* (Hedw.) Reich. was assessed in old forest stands (impacted by fragmentation) and more recently recovered forest stands (young). Three forest stands impacted by fragmentation were identified from aerial pictures of 1936 Puerto Rico; all stands were located in the Guajataca State Forest. Three forest stands that did not exist in the same location in 1936 were catalogued as young. Five populations were identified in each stand and three individuals collected per population. All samples were carried through the AFLP method for polymorphism identification. Mean within population diversity (Nei's H_s) was 0.257 while the proportion of total genetic diversity among fragments (Fst) was 0.199. When old and young forest stands were compared for within genetic diversity, based on the number of polymorphisms, there was no significant difference (1_O = 43; 2_O = 38; 3_O = 39; 1_Y = 38; 2_Y = 36; 3_Y = 32). The clustering analysis based on genetic distances (Fst) showed that Fragments 1_O, 1_Y, 2_O and 2_Y appear to be more closely related. Fragments 3_O and 3_Y appeared to be more genetically distant to all other sampled fragments.

RESUMEN

Los eventos de deforestación ocurren con rapidez en los países tropicales. La consecuencia de esto son paisajes formados por fragmentos de bosques entre áreas urbanas. La fragmentación en Puerto Rico ha demostrado tener efecto negativo sobre las comunidades de briofitos. Por que los briofitos son considerados plantas con habilidades de dispersión limitada y la fragmentación puede resultar en eventos de deriva génica que reduzcan la diversidad genética de poblaciones aisladas. La diversidad genética de poblaciones del musgo *Neckeropsis undulata* (Hedw.) Reich. fue estudiada en localidades de localidades de bosques viejos (impactados por la fragmentación) y localidades de bosque mas recientemente establecidos (jóvenes). Tres localidades de bosque impactados por la fragmentación fueron identificados a través de fotos aéreas de Puerto Rico tomadas en el 1936. Los fragmentos están localizados en el Bosque Estatal de Guajataca. Se catalogaron como jóvenes tres localidades de bosque que no existían en el 1936 en el mismo lugar. Se colectaron tres individuos de cada una de cinco poblaciones distintas para cada fragmento. Todas las muestras fueron procesadas a través del método de AFLP para la identificación de polimorfismos. La diversidad genética promedio intra-poblacional fue (H_s de Nei) 0.257 mientras que la proporción de diversidad genética total entre las poblaciones (Fst) fue 0.1990. Cuando las localidades viejas y jóvenes fueron comparados en términos de la diversidad genética intra-poblacional, basada en el número de polimorfismos, las diferencias no fueron significativa (1_O = 43; 2_O = 38; 3_O = 39; 1_Y = 38; 2_Y = 36; 3_Y = 32). El análisis de conglomerados basado en distancias genéticas (Fst) reveló que los fragmentos 1_O, 1_Y, 2_O y 2_Y están mas genéticamente

relacionados entre si. Los fragmentos 3_O y 3_Y parecen estar más genéticamente distantes de todos los otros fragmentos muestreados.

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INTRODUCTION

Social and political changes in Puerto Rico led to the clearing of 85% of its vegetation for agricultural purposes. This was followed by abandonment of farms and pastures and migration of human populations to urban areas due to an increase in manufacture driven by industrialization after the Second World War (Cruz-Báez and Boswell, 1997; Dietz, 1986, both cited by Grau et al., 2003). Puerto Rico's forests recovered after 50 years of intense agriculture (Grau et al., 2003); therefore, the landscape today is one of forests fragments scattered among urban areas (Lugo and Helmer, 2004). Grau et al. (2003) considered the rapid recovery, through succession, a result of the small size of the abandoned farms and the infrequent use of fire that avoided soil degradation. Furthermore, the abundance of forest remnants facilitated seed dispersal and tree establishment.

Most studies on the effects of fragmentation have been done on vascular plants, although Pharo and Zartman (2006) emphasized the unique qualities of many bryophyte taxa to evaluate the effects of habitat fragmentation. Among these are that bryophytes are widely distributed. In addition, they share phylogenetic similarities even among groups from different continents, allowing the evaluation of fragmentation impacts in different regions (Pharo and Zartman, 2006). Since bryophytes are easily impacted by changes in humidity, they are excellent models to study long term effects of microclimate change due to habitat fragmentation (Hylander, 2006). The short generation times of many bryophyte species represent an ideal characteristic to study the effects of fragmentation

on recently impacted areas such as those in the tropics or recently recovered landscapes like those of Puerto Rico.

Fragmentation clearly has had an effect on bryophyte diversity in Puerto Rico; for example, Escorcia (1998) found that forest fragments surrounded by densely urban areas in the San Juan metropolitan area of Puerto Rico showed a low bryophyte cover and lacked species typical for the temperature and humidity conditions found in the fragments. Species found by that author represent 8% of the total moss bryoflora on the island; 14% of the mosses found are also present in the Luquillo Experimental Forest. The most abundant species in these fragments were *Taxitelium planum* (Brid.) Mitt., *Neckeropsis disticha* (Hedw.) Kindb. and *Neckeropsis undulata* (Hedw.) Reich. The latter can survive in mesic habitats or with less relative humidity than sinkholes (Escorcia, 1998).

Forest fragment isolation represents a barrier to genetic flux. Genetic drift can reduce the genetic diversity of small and/or isolated populations, thus affecting the capabilities of a species to adapt to its changing environment and increasing the risk of extinction (Keller and Waller, 2002 cited by Wilson and Provan, 2003). Although *N. undulata* is drought resistant (Escorcia, 1998), it inhabits shade, where it evades desiccation and high temperatures (Reyes-Colón, 1999). Escorcia (1998) hypothesized that an epiphyte species like *N. undulata* colonizes through the dispersal of vegetative fragments and carries out sexual reproduction after its establishment. As for most of bryophyte taxa, studies about the dispersal abilities of *Neckeropsis undulata* have not

been conducted. Bryophytes are commonly characterized as having limited dispersal abilities. Spaces of centimeters between populations have been considered enough to interfere with genetic flux (Wyatt, 1982). Therefore, the expected consequences of fragmentation on *N. undulata* populations are those common to other bryophyte species; that is, genetic variation would be reduced by genetic drift acting on a bisexual species expected to have high levels of inbreeding due to self-fertilization. Crossing between haploid brother and sister plants produced by the same sporophyte is estimated to augment homozygosity by 90% in ten generations (Shaw, 2000).

This study evaluated the genetic diversity of populations of *Neckeropsis undulata* in old forest stands (forest that existed in 1936) and more recently recovered forest stands (forest stands that did not exist in 1936). Assuming that populations in young forest stands (did not exist in 1936) were founded by individuals in the surrounding forest remnants then the genetic diversity of the young forest stands would be a sample of the founder source. Therefore, I hypothesized that genetic diversity will be greater in old forest stands than in young forest stands. Given that bryophytes have limited dispersal abilities (Wyatt, 1982), I expect that stands that are geographically closer should also be genetically related. The genetic relationships established in this study may contribute to the knowledge of *Neckeropsis undulata* dispersal abilities. This study pretends to determine which forest stands carried the most genetic diversity and could be used for re-introduction purposes. Accordingly, the outcome of this study may contribute to develop a model for the restoration of an epiphyte moss species in a fragmented landscape.

METHODS

Species

Neckeropsis undulata is an epiphytic moss with a synoicous breeding system. This species inhabits branches and trunks of moist to lower mountain forests (Gradstein et al. 2001) and sometimes is also on logs and rocks. This species is distributed from Mexico to Brazil and is also present in Argentina and southern Florida.

In Puerto Rico, *Neckeropsis undulata* is a species common in the karst belt which begins in the northwest of the island, in Aguadilla, and ends at El Río Grande de Loíza, in the northeast. It occurs on haystack hills and in sinkholes in the Guajataca, Río Abajo and Cambalache forests (Reyes-Colón, 1999). Outside of the karst zone, it is also reported for the Luquillo, Maricao and Toro Negro forests (Sastre-D.J. and W.R. Buck, 1993). This species is also in forest fragments surrounded by disturbed areas like forests stands behind the Civil Engineering and Biology buildings at the University of Puerto Rico-Mayagüez. Escorcia (1998) found *N. undulata* in forest stands surrounded by densely urban areas.

Sampling

Forest stands

Forest stands that were present in Puerto Rico in 1936 were identified from a shape file (digital file) (J. D. Chinea, unpublished data). This data was visualized by opening the shape file over a digital topographic map (US Geological Survey) of the

Guajataca State Forest using Arc Map (ArcGIS software). Accordingly, three forest stands that existed in 1936 were identified and designated as old. The criterion for selecting the forest stands was that stands must be in sinkholes where *Neckeropsis undulata* is usually present. Using the same criterion, forest stands that did not exist in 1936 were identified and classified as young. The coordinates of all six forest stands (3 old, 3 young) were obtained using the same software (Table 1). The forest stands were located in the field using an eTrex® GPS from Garmin and USGS topographic maps showing the location of the selected forest stands. Coordinates were entered into the GPS instrument and the Puerto Rico Datum was selected. The location of all six sinkholes is shown in Figure 1.

The forest stands studied are located in the Guajataca State Forest (Figure 1). This subtropical moist forest (Ewel and Whitmore, 1973) has an area of 926 hectares and elevations from 150 to 300 m. The forest is located in northwestern Puerto Rico in the municipality of Isabela. Haystack hills intermingled with sinkholes (Department of Environmental and Natural Resources, 1976) characterizes the forest, which was established in 1943.

Populations

In this study, populations are epiphyte colonies separated by a few meters as established by Szweykowski, et al. (1981) as cited by Stenøien (2002). Five populations were chosen in each forest stand. Since bryophytes are commonly characterized as clonal organisms, three individuals were collected from different points in the colony to avoid

collecting potential clones. Because the growth form of *Neckeropsis* is shelf-forming it is difficult to determine what an individual is. A single moss plant may extend to distant points in a colony through a stem that is attached to the tree; therefore, removing the complete stem ensures that the same plant was not collected twice. Samples were placed in plastic bags, covered with paper bags (to avoid frost damage) and kept on ice. DNA extractions were performed on the same day or the day after.

DNA extractions

Ninety individuals were collected from the six sites (3 old; 3 young); 47 individuals were chosen randomly using a method from Bernoulli (Särndal et al., 2003). The method consisted of listing the individuals (labeled by forest stand and population they belong to) in an Excel worksheet and assigning a random number to each. Because three individuals were collected from each of the five populations in a stand, 0.6 was used as the criterion to exclude samples. Samples were excluded (not carried through the AFLP analysis) if its random number was less than or equal to 0.6. The 47 individual plants included were carried through a CTAB DNA extraction protocol.

The tips of the plants' secondary modules (branches shown in Figure 2) were cut and washed in 70% ethanol, quickly dried and separated in microtubes. Each individual was macerated in microtubes using autoclaved sand (Sea Sand from Fisher Scientific) and 600 µl of DNA extraction solution (7.5 ml of Tris HCl pH 8.0, 1.5 ml EDTA 0.5M, 10 ml NaCl 5M, 0.5 g CTAB, 1.125 ml mercaptoethanol, 7.5 g PVP and 30.25 ml ddH_2O). Samples were incubated at 65 °C in a water bath for 30 minutes; 600 µl of chloroform

iso-amyl alcohol (24:1) were added and mixed by immersion during 15 minutes. Samples were then centrifuged for 10 minutes in an Eppendorf 5415D centrifuge at 13,000 rpm. The aqueous phase was extracted with a micropipette, mixed with 300 µl of cold isopropanol and left in a (-20 °C) freezer overnight. On the following day, samples were centrifuged for 10 minutes in an Eppendorf 5415D at 13,000 rpm. Pellets were washed with 70% cold ethanol. DNA was then dissolved in TE (10 mM Tris-HCL, 1 mM EDTA, pH 8.0). Two microliters of RNAse (150 µl of TE: 5 µl of RNAse) were added to all DNA samples. DNA was quantified on 0.8% agarose gels with lambda molecular marker (0.5µg/µl; lambda digested with HindIII).

***AFLP* (*Amplified fragment length polymorphism*)**

All 47 DNA samples were carried through the AFLP four-step process using the Plant Genome AFLP Kit from LI-COR Biosciences.

Enzyme digestion

Samples, each containing 100 ng of DNA were digested in microtubes with 1 µl of *Eco*R1/*Mse*1 enzyme mix (1.25 U/µl in 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 200 µg/ml BSA, 50% (v/v) glycerol, 0.15% Triton X-100) and 2.5 µl 5X buffer (50 mM Tris-HCl pH 7.5, 50 mM Mg-acetate, 50 mM K-acetate). Water was added to complete a 12.5 µl reaction mixture. The mixture was incubated for 2 hours at 37 °C in an Eppendorf mastercycler, followed by an additional 15 minutes at 70 °C to inactivate the restriction enzymes.

Ligation

Adapters (LI-COR Biosciences) were tied to the fragmented DNA that resulted from the first AFLP step. Digested DNA (12.5 µl) was mixed with 2.5 U of T4 ligase (5U/µl en 10 mM Tris-HCL pH 7.4, 1 mM EDTA, 1 mM DTT, 50 mM KCL, 200µg/ml BSA, 50% (v/v) glycerol) and 12 µl of adapters mixture (Mse1 and EcoR1 adapters, 0.4 mM ATP, 10 mM Tris-HCl pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate); total reaction 25 µl. Adapters have terminals with sequences complementary to the sequence that the restriction enzyme recognizes and cuts in the DNA; thus, adapters have sequences that are complementary to the DNA fragments' terminals. Adapters are 20 nucleotides long, a known sequence from which a primer can be constructed: the aim of this step. The restriction/ligation mixture was incubated in an Eppendorf mastercycler at 20 °C for 2 hours. The mixture was later diluted 1:10 in TE (10mM Tris-HCl pH 8.0, 1 mM EDTA).

Pre-amplification

The pre-amplification cocktail had 2.5 µl of ligation dilution, 20 µl of primers (as prepared by LI-COR Biosciences for the AFLP plant genome kit), 2.5 µl 10X buffer and 0.5 µl (5 U/ µl) of Taq DNA polymerase (Roche Molecular Biochemicals). Pre-amplifications were carried out in 96-well Eppendorf plates. The cycle program was as follows: 20 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min in an Eppendorf mastercycler. The PCR reactions were diluted 1:40 in ddH_2O. The primers used in this step had a 5' end that was complementary to the adapter sequence and a 3' terminal with one arbitrary nucleotide. This nucleotide is known as a selective base because is meant to

reduce the amount of DNA fragments it amplifies. The fragments amplified must have matched the 3' end of the primer.

Selective amplification

The objective is to further select among the amplified fragments by adding three selective nucleotides to the primers. One of the primers was labeled with a fluorescent dye that allows its visualization after a run on a polyacrylamide gel. A total of 2.0 µl of pre-amplified and diluted DNA fragments was added to a cocktail containing: 4.79 µl ddH_2O, 1.21 µl 10 X buffer, 0.3 U of Taq DNA polymerase (Roche Molecular Biochemicals), 2.0 µl *Mse*1 primer containing dNTP's, 0.5 µl of *Eco*R1 fluorescent primer (LI-COR IR-Dye 700).

All 47 samples were selectively amplified with three different pairs of primers. Each pair differed in the selective nucleotides carried. Table 2 shows primer pairs identified by the restriction enzyme whose restriction site sequence it complements and the three selective nucleotides it carries.

Reactions were carried in 96-well Eppendorf plates. The touchdown PCR program cycles were as follows: one cycle of 94 °C for 30 s, 56 °C for 1 min and 72° C for 1 min; 12 cycles where denaturation and extension temperatures were preserved and the hybridization temperature descended 0.7° C each cycle; 23 cycles of 94° C for 30 s, 56° C for 30 s and 72° C for 1 min. The touchdown program was run in an Eppendorf mastercycler. Selective amplification was repeated for 10% of the samples, chosen randomly, to assure its reproducibility.

Electrophoresis

One microliter of the selective amplification product was denatured in 3 µl of blue stop solution (EDTA, blue bromophenol, ddH_2O and formamide in proportions (w/v%) of 1:1:14:87) at 94° C for 3 min in an Eppendorf mastercycler. Samples were immediately put on ice. The electrophoresis run followed immediately after denaturation in a 64-well, 0.25 mm thick polyacrylamide gel (KB^{Plus} 6.5%) in a LI-COR DNA Analyzer 4300. Run parameters were 1500, 40 W and current of 40 mA at a temperature of 45 °C. One microliter of the denaturation sample ran for 2 hours. The molecular marker (50-700 bp, LI-COR IR-Dye 700) was denatured at 95 °C for 2 minutes right before the run and placed in the first and last rows among samples.

Polymorphism visualization

Polyacrylamide gel images were analyzed through Cross Checker v 2.91 (J.B. Buntjer, 1999). The binary interpretation balance value was 5. Bands were aligned with the molecular marker. Binary (0, 1) data were exported to an Excel worksheet. The Data were rearranged so they could be introduced into the AFLP analysis software.

AFLP data analysis

Binary data was introduced as a text file into the AFLP-SURV 1.0 software written by Xavier Vekemans. Allelic frequencies were calculated through the fragment frequency method. The method assumes individuals are haploid and the dominant allele frequency is equal to the frequency of the AFLP marker (AFLP-SURV manual); 10,000 random permutations were performed to test the genetic differentiation among populations (F_{st}).

Similarly, genetic distance matrixes were bootstrapped 10,000 times. Lynch and Milligan (1994) methods for the analysis of population genetic structure were followed. The mean gene diversity within forest stands (H_w) and F_{st} values for population genetic structure were determined.

Genetic diversity within forest stands

Once the allelic frequencies were calculated through the fragment frequency method, the number of polymorphic markers and proportion of polymorphic markers for each forest stand were automatically calculated. A locus here is considered polymorphic if the frequency of one of its alleles is less than or equal to 0.95. Although, gene diversities based on Nei's Hj are more often used in literature, here the rate of polymorphism was preferred as a method to show variation at the locus level. The number of polymorphic markers was adjusted to correct for differences in the number of samples. The number of polymorphic markers (S) was divided by 1+1/2+1/3…(n-1), where n is the sample size (6 forest stands). A Kruskal-Wallis test was carried to determine if the differences in genetic diversity among old and young forest stands were significant.

Average gene diversities (Hj) are measured from the expected frequency of heterozygous genotypes for each locus; gene diversity here is the probability of finding one marker and one null in a locus (Lynch and Milligan, 1994). Average gene diversities (H_j) for each forest stand were presented in Appendix I. Standard error and variances due to sampling of individuals and loci were also presented.

Mean gene diversity (H_w) was calculated from the sum of averaged gene diversities (H_j) for each forest stand; the sum was divided by the number of stands (n=6). H_w is the mean within-population expected heterozygosity.

$$H_w = 1/n \sum_{j=1}^{n} \hat{H}_j \quad (1)$$

Mean gene diversity within forest stands was presented in this thesis for the purpose of comparing with previous studies of genetic diversity in mosses.

Genetic differentiation among forest stands

The impact of fragmentation on remnant populations and genetic status of the more recently established ones was also analyzed by measuring population subdivision using the following equation:

$$F_{st} = H_B / H_T$$

The component H_B is the average gene diversity for all pairs of populations and $H_T = H_B / H_w$. All measures of gene diversities are based on heterozygosity, where H_B is the heterozygosity (probability of finding one marker and one null in a locus) averaged for all distinct pairs of populations (stands) following the expression:

$$\hat{H}_B = \frac{2}{n(n-1)} \sum_{j<k} \hat{H}_{jk}$$

The heterozygosity for one locus (i) between two populations ($_{j,k}$) (stands) was defined by,

$$\hat{H}_{jk}(i) = q_j(i) + q_k(i) - 2q_j(i)q_k(i)$$

The heterozygosity for all loci between two populations (stands) was averaged from the expression,

$$\hat{H}_{jk} = 1/L \sum_{i=1}^{L} \hat{H}_{jk}(i)$$

The measure of population subdivision (F_{st}) takes extreme values of zero when all populations have identical gene frequencies (no divergence) and one when there is fixation of alternate alleles in different subpopulations (complete divergence). A value of 0.2 for F_{st} means large genetic differentiation among populations or as in this study among forest stands. Wright's F_{st}, used in this study with haploid data, measures the correlation between pairs of markers randomly sampled within a population relative to pairs of markers randomly sampled within the overall set of populations as established in the AFLP_SURV manual. Also, 10,000 permutations were made to test the F_{st} value observed against the distribution obtained by permutation. The null hypothesis is that there is no genetic differentiation among stands.

Genetic distances among forest stands

A dendrogram was constructed from an F_{st} genetic distance matrix. The genetic distance matrix calculated by the AFLP_SURV software was introduced into the free software T-REX 4.0a1 (2000), written by Vladimir Makarenkov. Nei and Saitou's (1987) neighbor-joining method was selected to construct the tree. The length of a branch in the dendrogram represents a genetic distance. Allele frequencies in forest stands that are not outcrossing will differ from the allele frequencies of the overall set of populations; hence genetic distances would be greater among forest stands without genetic flow among them.

Genetic distances among individuals

A matrix of pairwise genetic distances calculated by AFLP_SURV software was introduced into the T-REX 4.0a1 software. Nei and Saitou's (1987) neighbor-joining method was selected to construct the tree. The length of a branch in the dendrogram represents a genetic distance and the dissimilarities among individuals. Relationships among individuals were also represented through a principal coordinates analysis (PCoA). The software NTSYS 2.0, written by F. James Rohlf, was used for PCoA. Similarity was measured using the Jaccard index. The Jaccard similarity coefficient measures the number of attributes (markers) that individuals share relative to the overall set of attributes. The attributes positively (1) or negatively (0) observed for each individual are considered the individuals' coordinates. Double absence is not counted to avoid overestimating the similarity by using characteristics that are not

present. An Eigen analysis was carried after double centering the similarity matrix. Eigen values are a measure of the amount of variation along the axis. The closeness among points in the PCoA approximate the similarities among individuals sampled.

RESULTS

Genetic diversity within forest stands

The proportion of polymorphic makers was greater for forest stand 1_O (43%) and was the smallest for 3_Y (32%) (Table 3). The same number of variants were found for old stand 2_O and young stand 1_Y (2_O = 38; 1_Y = 38). Although the quantification of genetic diversity within the forest stands based on the number of polymorphic markers showed a larger number of variants in the old forest stands (1_O = 43; 2_O = 38; 3_O = 39) than in the younger forest stands (1_Y = 38; 2_Y = 36; 3_Y = 32) these were not significantly different (p = 0.2, Table 4). The mean genetic diversity within forest stands determined by Nei's H_w was 0.257.

Genetic differentiation among forest stands

Genetic differentiation was large among forest stands with an F_{st} of 0.199 (Table 5). The standard error for this index was 0 and variance was negative and thus meaningless. Permutation tests resulted in the rejection of the null hypothesis that there is no genetic differentiation among the forest stands (Table 5). When the F_{st} value was compared against its distribution it resided at the 5% rightmost part of the distribution under the null hypothesis. The result of the P value (high) was equal to 0. The P value (high) is the probability of rejecting a true null hypothesis as a one sided test with the observed F_{st} higher than values under the null hypothesis (AFLP_SURV manual). From these results it is concluded that actual populations of this plant are more genetically differentiated than random samples of the individuals (AFLP_SURV manual).

Genetic distances among forest stands

The dendrogram (Figure 3) formed two clusters based on dissimilarities of marker frequencies among the studied forest stands. The most genetically distant cluster was number *II* formed by stands 3_O and 3_Y with a distance of 0.14. Cluster *I* was formed by stands 1_O, 2_Y and 1_Y with a distance of 0.2. Inside cluster *I*, stands 1_O and 2_Y grouped apart from 1_Y with a distance of 0.01. Stand 2_O formed an individual cluster with a distance of 0.04.

Genetic dissimilarities among individuals

The dendrogram (Figure 4) formed four clusters based on the divergence of the individuals. The largest cluster named *I*, included all individuals collected from stand 2_O (2_O1, 2_O2, 2_O3, 2_O3, 2_O4, 2_O4, 2_O5 and 2_O5) apart from one of the individuals collected from population number one (2_O1). Cluster *I* also grouped all individuals from stand 2_Y (2_Y1, 2_Y2, 2_Y3, 2_Y3, 2_Y4, 2_Y5 and 2_Y5) except for one individual collected from population number one (2_Y1). Five of the seven individuals collected from stand 1_Y belong to cluster *I* also: 1_Y1, 1_Y1, 1_Y4, 1_Y4 and 1_Y5. A small group of individuals collected from stand 1_O belong to cluster *I*: 1_O1, 1_O4 and 1_O5; a total of nine were collected from this forest stand. One individual (3_O5) from stand 3_O fell into cluster *I*. Also, one of the individual (3_Y4) from forest stand 3_Y was grouped into cluster *I*.

All individuals collected in stand 3_Y were grouped in cluster *II* (3_Y1, 3_Y2, 3_Y2, 3_Y3, and 3_Y4) with the exception of one individual from population four (3_Y4). Also

inside this cluster six of eight individuals collected from stand 3_O: 3_O1, 3_O2, 3_O3, 3_O3, 3_O4 and 3_O4. This cluster showed the most divergence with a genetic distance of 0.07.

Cluster *III* included three individuals from the same forest stand and population 1_O2. The group also included two other individuals from forest stand 1_O: 1_O3 and 1_O1. Individuals 1_Y5 and 1_Y5, two of three individuals collected from forest stand 1_Y, were group into cluster *II*. The smallest cluster (*IV*) incorporated three individuals: 3_O2, 2_Y1 and 1_O1 and was the second most dissimilar with a genetic distance of 0.06.

Principal coordinate analysis

Similarities between the individuals sampled were represented through a principal coordinate analysis based on a Jaccard similarity index (Figure 5). A closely related group was formed by eight individuals collected from forest stands 3_O and 3_Y (3_O2, 3_O3, 3_O3, 3_O4, 3_Y1, 3_Y2, 3_Y2 and 3_Y3). Closely related here implies that they share a great number of markers relative to the overall set of markers sampled. All individuals sampled from forest stands 1_O and 1_Y formed a group of more distantly related individuals. This group is larger and it also includes all individuals from forest stands 2_O and 2_Y apart from: 2_O1, 2_O1, 2_O3 and 2_Y3. The group also included one individual from stand 3_O (3_O5).

DISCUSSION

The differences in genetic diversity within forest stands (old and young) were not ample. When old and young forest stand pairs were compared, the number of polymorphisms was to some extent higher in the older pair (Table 3). However, the differences in genetic diversity were not significant (Table 4). The genetic diversity analysis measured through polymorphisms and the Nei's H_j (Appendix I) revealed that the genetic diversity was the highest in stand 1_O and the lowest in stand 3_Y (Table 3; Appendix I). Mean within-population gene diversity (Nei's H_w) found for *Neckeropsis undulata* (0.257) is comparable to but somewhat higher than values found in other epiphytic mosses genetic diversity studies using AFLP markers. Snall (2004) measured the genetic diversity of two epiphytic species, the monoicous *Orthotrichum speciosum* and the dioicous *Orthotrichum obtusifolium*; and the mean gene diversity within populations was 0.19617 and 0.19653, respectively. On the other hand, Zartman's (2006) values for *Radula flaccida*, an epiphyllous Amazonian liverwort, were higher for populations of this species in forest remnants and continuous forest (0.412±0.2 and 0.413±0.2, respectively).

Young forest stands were expected to have less genetic diversity than older stands, because of possible limited dispersal as it is traditionally viewed for other bryophyte taxa. The traditional view is that distances of a few centimeters are enough to obstruct gamete dispersal. Gamete dispersal for an epiphytic species may extend beyond two meters (Wyatt, 1982). *Neckeropsis undulata* produces a great number of sporophytes especially

during the rainy season in Puerto Rico (extending from May to November; Daly et al., 2003) as has been observed during field work by our bryology group. Consequently, the plant is expected to reproduce at least once a year. If populations in these young forest stands (forest that did not exist in 1936) were established by founder events then the genetic diversity within them would be a sample of the genetic diversity of surrounding remnants (founder source). The impact of fragmentation on old forest stands (forest fragments that existed in 1936) may no longer be evident in the genetic diversity measured by marker frequencies. Since Puerto Rico's forests are expected to recover in 50 years (Grau et al., 2003) and the Guajataca State Forest was designated in 1943, then, if genetic drift operated over populations of *Neckeropsis undulata* in old forest stands the plant had at least 70 generations (70 years) till this study to recover from the drift event. When the young forest stands studied did start to emerge is not a question answer in this study.

The relationships found among forest stands and individuals sampled may explain the genetic diversity found. The forest stands studied were highly differentiated (F_{st} = 0.199) with respect to marker frequencies. Values found in this study are lower than that reported in Hassel et al. (2005) who found F_{st} of 0.223 among populations of the moss *Pogonatum dentatum* in a mountain area and in a more recently colonized area from Sweden. The average value of genetic differentiation among moss populations is 0.234 (Korpelainen et al, 2005).

Assuming that the expanding ability of *N. undulata* is limited and considering that forest stand pairs (old and young) were geographically closer to each other, each young forest stand was expected to be more closely related to its older pair. The cluster analysis (Figure 3) based on genetic distances (differences in marker frequencies) showed that this relationship was true between forest stands 3_Y and 3_O but not as expected for stands 1_O, 1_Y, 2_O and 2_Y. Forest stands 3_O and 3_Y were the most genetically distant as a group from all other stands. Gene flow may explain the relationship demonstrated among forest stands: 1_O, 1_Y, 2_O and 2_Y. The cluster analysis based on genetic differences among the individuals sampled (Figure 4) revealed that the majority of individuals collected from these forest stands formed a large cluster (Figure 4; cluster *I*). The relatedness among these individuals is also represented in the PCoA where the majority of individuals from stands 3_Y and 3_O formed a well defined cluster of closely related points whereas individuals collected from the remaining forest stands formed a less defined group.

Gene flow among forest stands 1_O, 1_Y, 2_O and 2_Y along a forest trail may explain the non-structured relationship among these stands and the individuals sampled from them. Hassel et al. (2005) suggested that forest roads were a window for vegetative diaspores to expand from a mountain to a lowland area and explained the levels of genetic diversity found in a newly colonized area through sexual reproduction. Forest stands 1_O, 1_Y, 2_O and 2_Y are connected by one of the most hiked trails in the Guajataca State Forest while forest stands 3_O and 3_Y are more isolated. Reaching stands 3_O and 3_Y required leaving a trail and hiking over hills and through sinkholes

(high and low elevations). Figure 6 shows two forest trails: trail A was a forest road present in the forest in USGS maps from 1972 and Trail B represents three forest trails present in the forest today that connect the studied forest stands. Trail B also connect a large camping area next to forest stands 1 and the most visited place in the forest: "La Cueva del Viento" which is close to forest stands 2. Today, *Neckeropsis undulata* vegetative fragments may be carried to different points in the forest by forest campers and/or visitors. As Escorcia (1998) hypothesized, founders in the form of vegetative fragments possibly reached the forest stands and expanded through sexual propagation once established. Escorcia (1998) observed that while epiphylls disappeared from the disturbed forest remmants, epiphytes moved down on its ecological niche. Although, populations of *Neckeropsis undulata* are commonly found in the base of tree trunks Escorcia observed most colonies over rocks. Although, the dispersal methods used by *N. undulata* have not been studied directly; new findings in laboratory cultures of *N. undulata* (C. Pasiche and I. Sastre-D.J unpublished data) suggest that the plant's establishment methods may go beyond the commonly view ones (spores/vegetative fragments). Brood cells were observed in laboratory trials when media nutrients were decreasing (Figure 6). These cells have been found as an inconspicuous stage in the life cycle of many bryophyte taxa and are commonly produced during desiccation (Mallón et al., 2006). The spherical thick wall cells, observed by Mallón et al. (2006) in *Splachnum ampullaceum* Hedw., were also observed in *N. undulata* when cultivated in 0.5x Murashige and Skoog media (Figure 7). Mallón et al. (2006) hypothesized that brood cells were part of propagule soil banks and because they remain viable even when desiccated, the cells may be part of the plants' reproductive strategy to survive

disturbances. In order to establish how the populations in the young forest stands were founded, it is necessary to identify the dispersal strategies of *Neckeropsis undulata* and the presence of these asexual propagules in soil banks, tree bark and rocks.

The common assumptions very limited dispersal abilities and lack of diversity attributed to bryophytes were rejected by Korpelainen et al. (2005) based on results of most genetic differentiation studies and the patterns of gene flow inferred from them. Bryophytes may be considered anatomically and physiologically primitive; nevertheless, their reproductive strategies appear to be successful. Populations of *Neckeropsis undulata* have shown great levels of genetic diversity after a well documented event of deforestation on the island.

CONCLUSIONS

Populations of *Neckeropsis undulata* are present today in forest stands that were completely deforested in 1936 with genetic diversity not different from populations of older forest stands. The question remains whether the plant survives deforestation by colonizing rocks as observed by Escorcia and if the shade provided by the sinkhole allowed the plant to be successful in using this strategy or if populations emerged from brood cells present in soil banks or perhaps the rocks. Although the specific reproductive strategies of this species have not been identified; *Neckeropsis undulata* populations' strategies appeared to be successful in maintaining diversity comparable to other bryophyte taxa and showed genetic relationships among geographically distant populations.

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TABLES

Table 1. Decimal coordinates for the studied forest stands. Forest stand pairs were numbered (1-3) and identified as old or young through the letters O and Y. Coordinates were obtained from ArcMap (ArcGis software)

Forest stands	Decimal latitude	Decimal longitude
1_O	18.42816	-66.97902
1_Y	18.42729	-66.97837
2_O	18.41092	-66.97879
2_Y	18.41158	-66.97680
3_O	18.41059	-66.98783
3_Y	18.40874	-66.98600

Table 2. Pairs of primers used in the selective amplification. Each Primer is named based on the first letter of the restriction enzyme whose restriction site sequence it complements and the three selective nucleotides it carries.

Primer pair	Mse1	EcoR1 (IR-Dye)
I	M-CAT	E-ACT
II	M-CAC	E-AAG
III	M-CTC	E-AAC

Table 3. Genetic diversity found within forest stands based on the number of polymorphic markers. The number of polymorphic markers was adjusted (Watterson's Θ) to account for the differences in the number of samples between stands.

Forest stands	Number of individuals	Total number of markers	Polymorphic markers	S/1+1/2+1/3…(n-1)	% of polymorphic markers
1_O	9	150	115	43	28.67
1_Y	7	150	91	38	25.33
2_O	9	150	102	38	25.33
2_Y	8	150	92	36	24.00
3_O	8	150	99	39	26.00
3_Y	6	150	71	32	21.33

Table 4: Results of Kruskal-Wallis test

Variable	forest stands	n	Means	S.D.	Average	H	p
diversity	old	3	40.00	2.65	39.00	3.05	0.2000
diversity	young	3	35.33	3.06	36.00		

Table 5. Genetic differentiation and permutation test results. Permutation test results are presented in the last three rows. If the value of F_{st} observed is lower than the value of *lower 95% limit,* the null hypothesis is rejected as a two sided test and it is concluded that the actual populations are more similar than random samples the individuals. When the value of F_{st} observed is higher than the value of *upper 95% limit,* the null hypothesis is rejected and it is concluded that the actual populations are more differentiated than random samples of the individuals. *P value (high)* gives the probability of rejecting a true null hypothesis as a one sided test.

Statistic	**Values**
observed F_{st}	0.1990
lower 95% limit	-0.0084
upper 95% limit	0.0503
P value (high)	0.0000

FIGURES

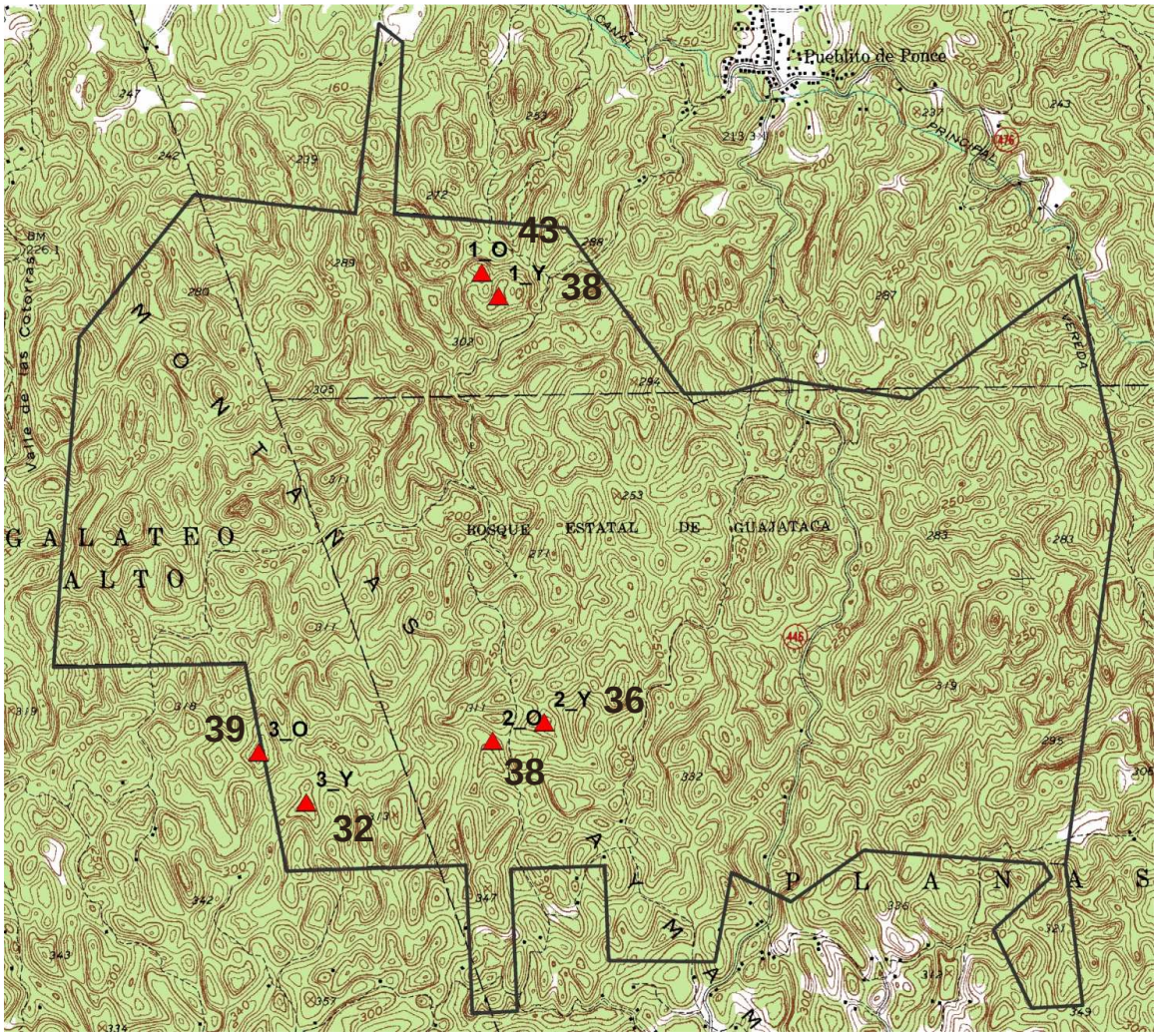

Figure 1. The image shows forest stand sites identify by name: 1_O, 1_Y, 2_O, 2_Y, 3_O and 3_Y. The number close to the forest stand name is the genetic variability (polymorphisms). The black outline shows the limits of the forest.

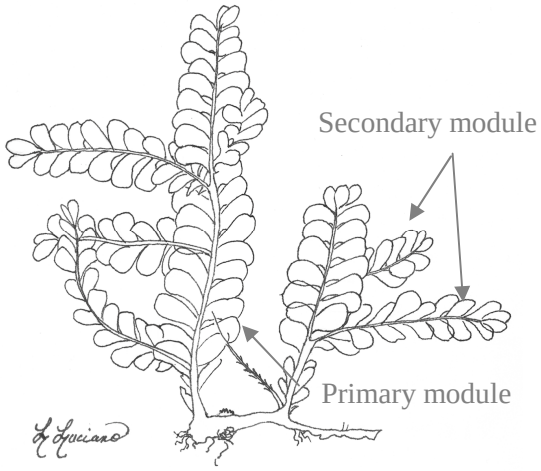

Figure 2. Drawing of *Neckeropsis disticha* showing module ramification.

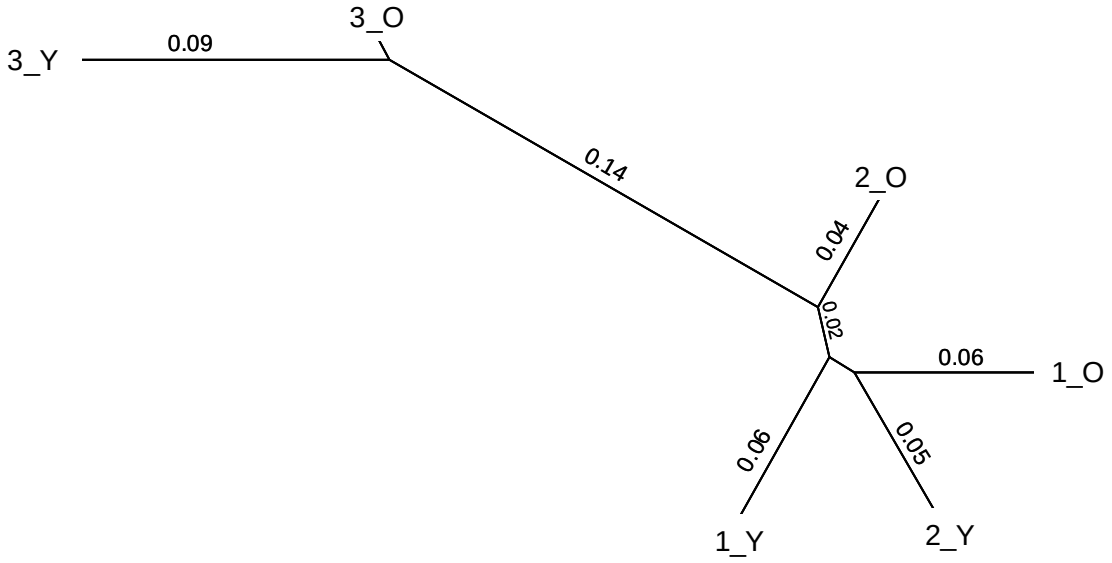

Figure 3. Cluster analysis (dendrogram) based on the genetic distances found among forest stands. The tree branches were identified by the names of the forest stands. Forest stands were named by numbers (1-3) and identified as old or young by the letters O and Y separated by an underscore. The length of a branch in the dendrogram represents genetic distance. The value of the genetic dissimilarity is shown along the branch. Distances were calculated through the F_{st} index. F_{st} is a measure of how much the forest stands differed in terms of marker frequencies. If there is no genetic flow between two forest stands their allele frequencies should differ and genetic distances would be greater among them.

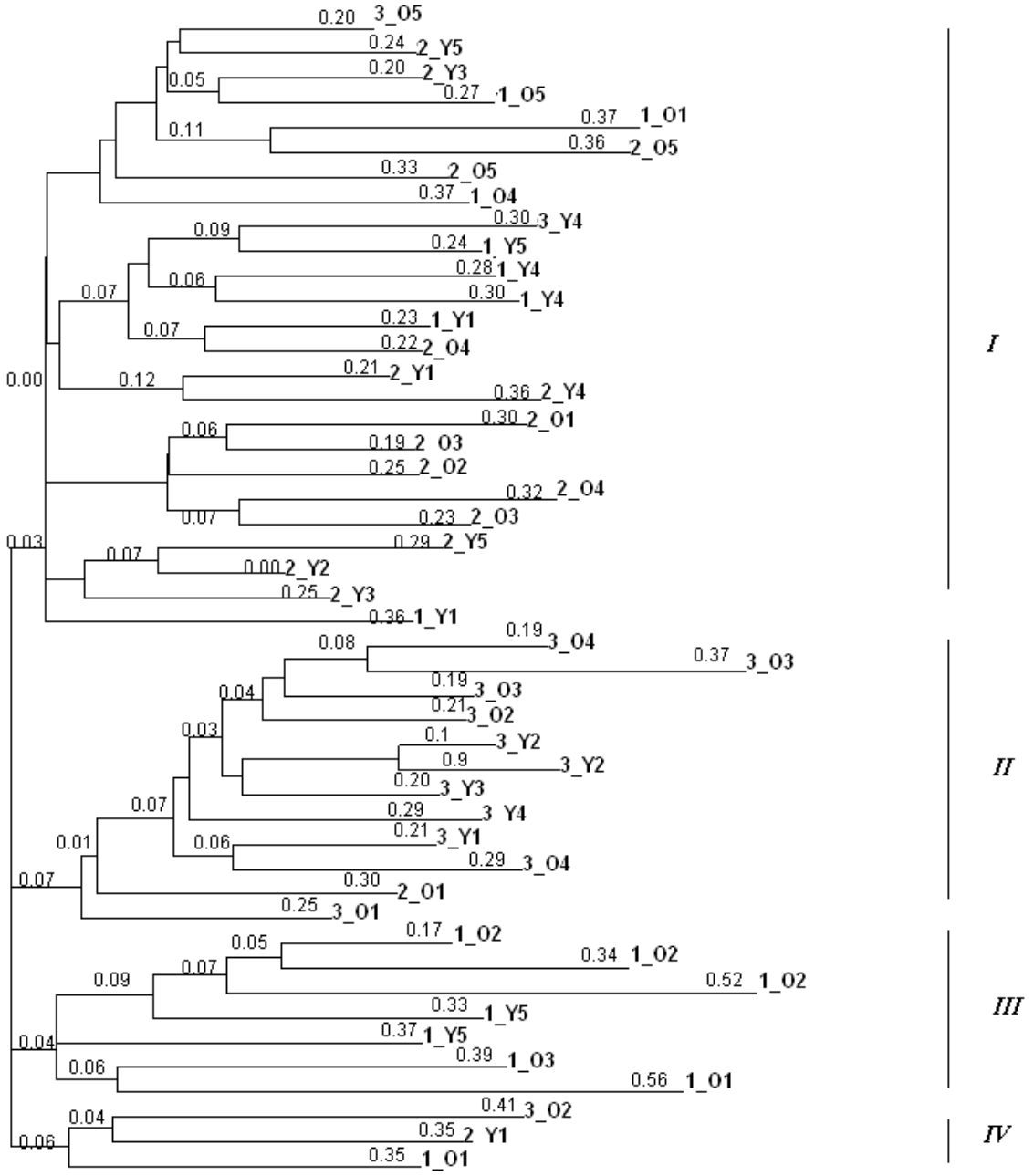

Figure 4. Cluster analysis (dendrogram) based on the genetic divergence found among individual plants. The tree branches were identified by the names of the forest stands and the population from which the individual plant was collected. Forest stands were named by numbers (1-3), identified as old or young through the letters O and Y separated by an underscore and followed by the number of the population (1-5). The length of a branch in the dendrogram represents a genetic distance. The value of the genetic dissimilarity is shown along the branch. Roman numbers reveal where clusters were formed.

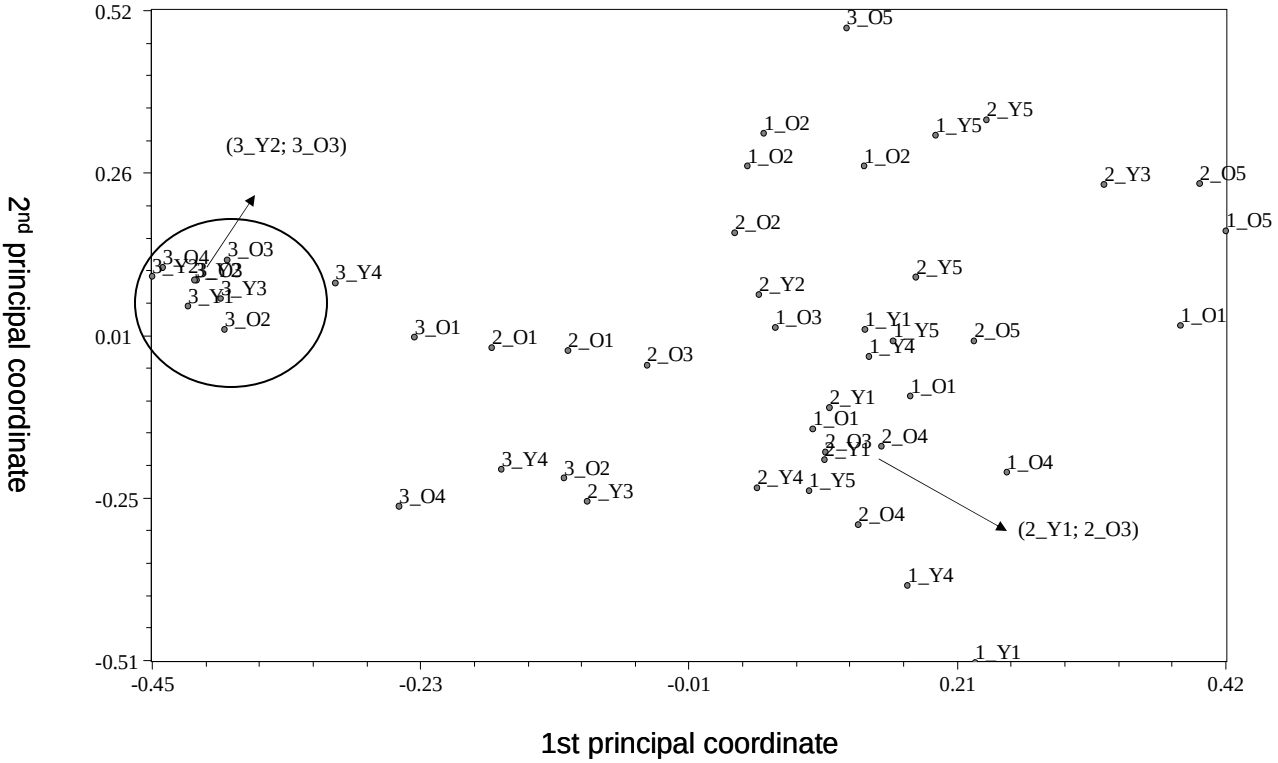

Figure 5. Principal coordinates analysis based on a similarity analysis (Jaccard index) among the individuals. Closeness between points in the graphic approximate the similarities among individuals sampled. The percentage of variability explained by the PC1 is 11.8% and the PC2 explained 21.4%. The circle shows a cluster formed by individuals from forest stands 3_O and 3_Y. The arrows points to individuals' names when names are not legible.

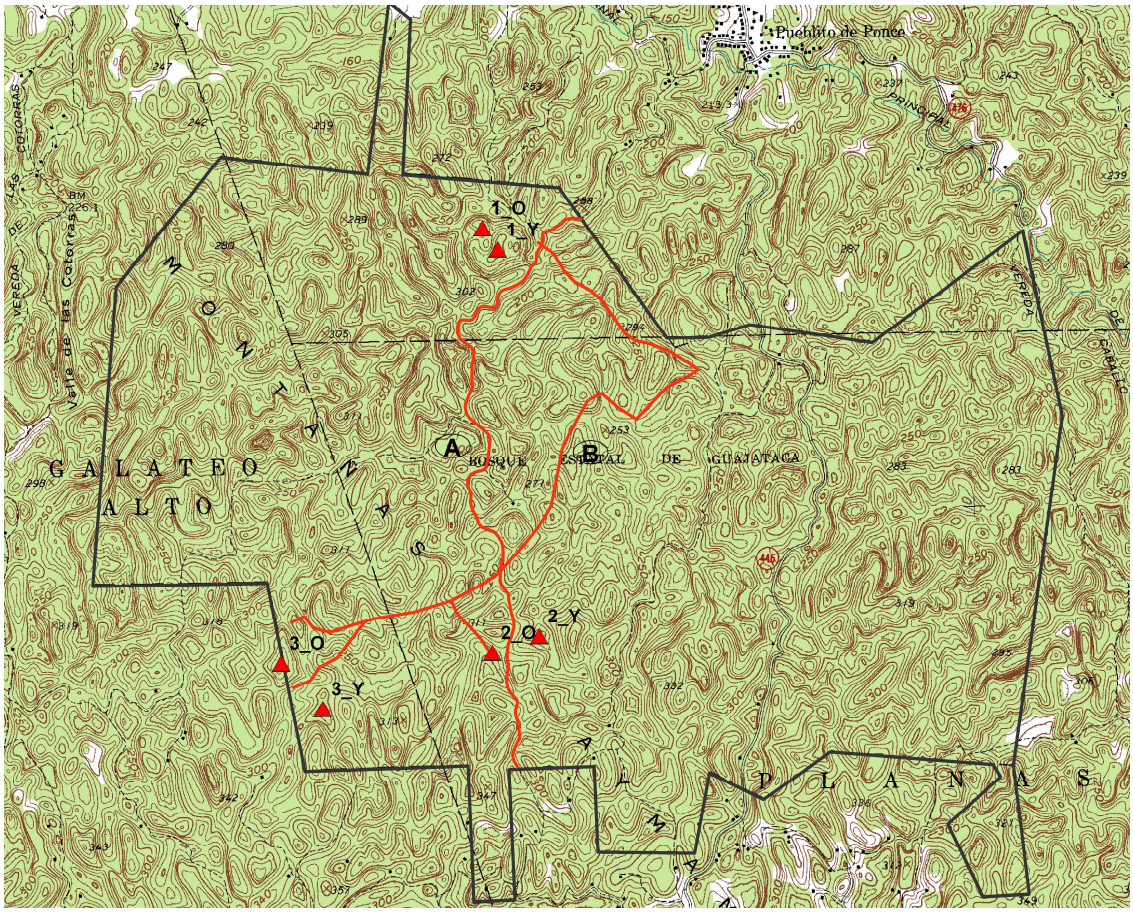

Figure 6: Forest trails connecting forest stands in the Guajataca Forest. Forest trail A was a forest road present in a USGS map from 1972. Forest Trail B represents three trails that connect forest stands 1 and forest stands 2.

Figure 7. Images of brood cells found in *Neckeropsis undulata* cultures (C. Pasiche and I. Sastre-D.J unpublished data). The photos were taken by José Almodóvar using a compound microscope.

APPENDICES

Appendix I. Results of the genetic diversity within forest stands measured through Nei's H_j. The statistics of H_j presented are: standard error [S.E. (H_j)], variance of H_j [Var(H_j)], variance component of H_j due to sampling of individuals [VarI(H_j)], proportion of variance of H_j due to sampling of individuals [VarI%], variance component of H_j due to sampling of loci [VarL(H_j)] and proportion of variance of H_j due to sampling of loci [VarL%].

Forest stands	H_j	S.E.(H_j)	Var(H_j)	VarI(H_j)	VarI%	VarL(H_j)	VarL%
1_O	0.30837	0.01686	0.000284	0.000081	28.6	0.000203	71.4
1_Y	0.25874	0.01856	0.000344	0.000086	25.1	0.000258	74.9
2_O	0.27032	0.01753	0.000307	0.000075	24.4	0.000232	75.6
2_Y	0.24352	0.01784	0.000318	0.000080	25.1	0.000238	74.9
3_O	0.25594	0.01708	0.000292	0.000092	31.6	0.000199	68.4
3_Y	0.20525	0.01885	0.000355	0.000080	22.6	0.000275	77.4