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Research Article

Incorporating molecular genetics into remote expedition fieldwork

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Abstract

Conservation expedition groups that use volunteer researchers are widespread in the United Kingdom and are growing in popularity around the world. These expeditions operate in regions of high biodiversity to study and protect the endemic species of these areas. New products have now made it possible to conduct molecular analyses in the field. We tested this in a volunteer-based conservation expedition to an area of tropical montane rainforest and cloud forest in Cusuco National Park, Honduras. Specifically, we (1) tested and modified recommended protocols for use of the new molecular techniques on a wide range of plant and animal species in the field, (2) tested the ability of novice volunteers to successfully use these techniques after minimal introductory training, and (3) used the novel techniques to conduct a small-scale population genetic study of *Liquidambar styraciflua* L. while on expedition. We found the techniques to be effective on all plant and animal species tested, with some modification of manufacturers' protocols. We also found that novice student volunteers were able to learn the required theory and protocols for the new technology, collect reliable data, and perform basic genetic analyses in a week-long DNA field sampling course. Finally, the *Liquidambar* case study demonstrated that genetic analyses can be successfully completed in primitive field conditions. These findings have exciting implications for work that can be done in remote locations, often areas of the greatest conservation significance.

Keywords: conservation volunteers, DNA field sampling, Honduras, *Liquidambar*, Whatman FTA cards

Resumen

Grupos de expedición de Conservación que utilizan investigadores voluntarios se han generalizado en el Reino Unido y están creciendo en popularidad en todo el mundo. Estas expediciones operan en regiones de alta biodiversidad y de trabajo para estudiar y proteger las especies endémicas de estas áreas. Los nuevos productos han hecho posible que hacer de los análisis moleculares en el campo. Pusimos a prueba esta en el contexto de una expedición de voluntarios de conservación en un área de bosque tropical y bosque nublado montano tropical en el Parque Nacional Cusuco, Honduras. Específicamente, (1) hemos probado y modificar los protocolos recomendados para el uso de las nuevas técnicas moleculares en una amplia gama de especies de plantas y animales en el campo, (2) hemos probado la capacidad de los voluntarios novatos para utilizar con éxito estas técnicas después de la formación inicial mínima y (3) se utilizó las nuevas técnicas para intentar un estudio genético de la población en pequeña escala de *Liquidambar styraciflua* L., mientras que en la expedición. Encontramos que las técnicas sean eficaces en todas las especies de plantas y animales han sido evaluados, con algunas modificaciones de los protocolos de los fabricantes. También se encontró que los estudiantes voluntarios novatos pudieron aprender la teoría y los protocolos necesarios para la nueva tecnología, recoger datos fiables, y hacer análisis genéticos básicos dentro de un curso de campo del ADN de una semana. Por último, el estudio de caso *Liquidambar* demostró que el análisis genético puede completarse con éxito en el campo primitivo. Estos resultados tienen implicaciones interesantes para el trabajo que se puede hacer en lugares remotos, y las áreas de mayor importancia para la conservación.

Palabras clave: voluntario de conservación, muestreo de campo del AND, Honduras, *Liquidambar*, Tarjetas FTA Whatman

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Introduction

Genetic information is increasingly important to all areas of biology, including ecology and biogeography, and is highly relevant to conservation [1]. As more conservation theoretical work incorporates genetics, conservation practitioners in expedition groups increasingly require more information about the genetics of the organisms they work with. Therefore, the goal of this study was to develop effective and inexpensive methods for novice expedition volunteers to perform accurate genetic analyses while in remote locales. To test the efficacy of this integrative approach to conservation biology, a small-scale study was conducted on expedition in Central America to estimate the genetic variability within and among *Liquidambar styraciflua* (American sweetgum) meta-populations. American sweetgum is an appropriate focal species because of its distribution throughout cloud forests of Mesoamerica, and large individuals are the target of illicit logging practices.

Mesoamerica, from central Mexico to Panama, is a biodiversity hotspot for floral and faunal species, with species endemic to this area comprising 1.7% of the world's plant species and 4.2% of the world's vertebrate species [2,3]. Despite the creation of over 270 wildlife protection areas in Central America between 1970 and 1987, these unique species are increasingly threatened by human activities [4]. The biodiversity of hotspots like Mesoamerica may be best protected via long-term monitoring and management programs. Long-term records provide conservation biologists with data that can be used to make informed conservation decisions, enabling more effective policies than those based only on one-off, transient studies [5].

Operation Wallacea (www.opwall.com) is an organization that operates scientific, volunteer-based wildlife conservation expeditions to regions of high biological diversity around the world, including Cusuco National Park, Honduras, and several other parts of the Neotropics. The professional scientists leading the expeditions conduct long-term, monitoring-based studies of major taxa (including insects, mammals, amphibians, plants and birds), with all the data feeding into a central database. Most of the paying volunteers on the expeditions are students, keen to learn new techniques. Volunteers are an increasingly used resource in conservation. In 2005, 7,550 volunteers participated in the expeditions of 19 UK conservation-based organizations [6]. With prior planning and proper training, volunteers collect reliable data quickly and with little cost to principal investigators [7-10].

In spite of the limitations of remote field expeditions, new and relatively inexpensive technology has made several molecular genetic procedures possible to implement in the field, including Whatman FTA® cards for DNA storage, a PCR bead for amplification, and the Lonza FlashGel System visualization. Whatman FTA® products are innovative storage systems for nucleic acids that can be used to store a variety of sample types indefinitely at room temperature. Mbogori et al. [11] successfully amplified genomic DNA after 14 years of storage on FTA cards, which withstand harsh conditions and are safe from fungal, bacterial, and viral contamination [12]. Blood samples are frequently stored on FTA cards [13-15], and DNA/RNA has been successfully amplified after storage on them [16-19].

Another valuable resource, the PCR bead (e.g. Illustra PuReTaq Ready-To-Go PCR beads) allows Polymerase Chain Reactions (PCRs) to be performed in field laboratories. PCR beads are stable at room temperature and contain all the reagents required for a successful PCR except water, primers, and template DNA. Successful amplifications have been performed using PCR beads for both animals [20] and plants [21]. For DNA electrophoresis, Lonza's FlashGel System contains cassettes and a dock. Cassettes are sealed, premade agarose gels, including stain and buffer, which can be stored at room temperature for up to 5 months (manufacturers' guidelines). The dock provides charge separation for gel electrophoresis; it contains a built-in illuminator for viewing the bands on the gel and has been successfully used with both genomic DNA [22] and plasmids [23].

Together, these products allow routine, rapid, on-site molecular genetic analysis in remote, difficult-to-access locations. To test the application of this new molecular genetic technology in a field environment, a small-scale study was conducted on an expedition in Honduras to examine genetic variability among and within the *Liquidambar styraciflua* meta-populations of Cusuco National Park, Honduras. *Liquidambar styraciflua* is a tree of the Altingiaceae family that has a disjunct distribution across much of eastern North and Central America. *Liquidambar* is often used as a case study for intercontinental disjunctions [24].

Integration of molecular techniques with fieldwork allows expedition biologists to investigate the genetics of species while on site in remote areas. The aim of the present study was to (1) introduce molecular techniques to conservation expedition volunteers by conducting a DNA field sampling course in conjunction with Operation Wallacea, (2) test and modify existing protocols of innovative molecular tools for effective use in primitive field conditions, and (3) conduct a small-scale population genetic study in the field with Operation Wallacea volunteers using the new molecular techniques described.

Methods

Study Site

Cusuco National Park (hereafter CNP) is a 23,400-ha protected area in the Merendon Mountains of Honduras, centred on 15° 32' N, 88° 15' W (Fig. 1). It ranges from near sea level to above 2,200m and comprises a 7,690-ha, high-altitude core zone surrounded by a 15,750-ha buffer zone of lower altitude. The park includes several main habitat types, including lower and upper montane rainforest, pine forest, and cloud forest. It supports important biodiversity, including six amphibian and seven reptile species known only from CNP and the newly described tree genus *Hondurodendron* [25], also endemic to the park. It is part of the Meso-American biodiversity hotspot, and was recently ranked as the 25th most irreplaceable of all the world's 173,461 protected areas (terrestrial, aquatic and marine) for conserving threatened amphibian species [26].

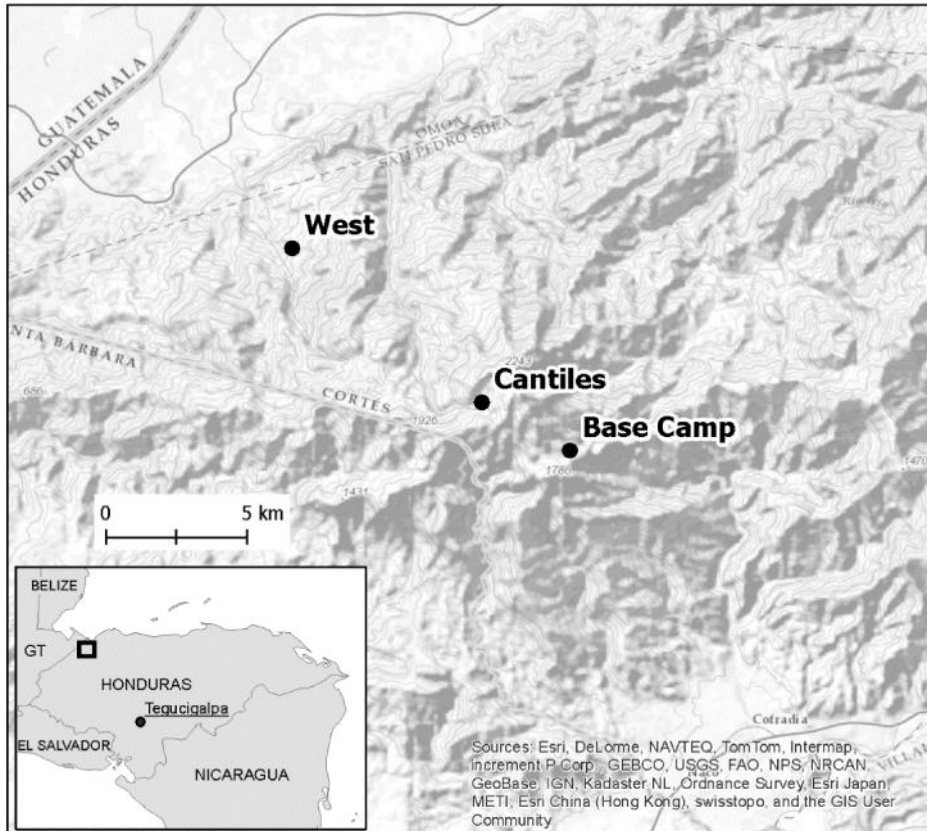


Fig. 1. The DNA field lab was located at Base Camp in Cusuco National Park, Honduras. The sample locations of American sweetgum (*Liquidambar styraciflua*) used in the population genetic study are identified by the black dots.

Field and Laboratory Methods

All plant and animal sample collection, DNA extraction, inter-simple sequence repeat (ISSR) amplification, and analyses were performed by Operation Wallacea volunteers in summer 2007, in a small field laboratory in CNP. The volunteers were all novice students and scientists, with no previous training in sample collection or molecular techniques, who participated in a week-long, on-site DNA field sampling course. Fifty volunteers were trained in sample collection and in the use of the molecular tools being tested. Their work was supervised by experienced scientists.

All procedures were performed in what is normally the CNP visitor center (1,546 m altitude). The molecular tools used are affordable to many expedition budgets and are easily obtained from the manufacturers. All equipment, including the Eppendorf small centrifuge and Mastercycler[®] PCR machine, Lonza FlashGels, and the computer used to run the gel imaging program, is portable and was brought to Honduras in airline luggage. The equipment was powered by a generator that operated for five hours each day. No refrigerator or freezer was available. Extracted DNA samples and ISSR primers were stored at temperatures of the surrounding montane rainforest (15°–30°C; materials were stored in a cool room in the CNP visitor center).

Volunteers collected leaf samples of seven plant species commonly found in CNP (Table 1). DNA from a portion of leaf tissue from each plant was transferred to Whatman FTA Plant Cards. Each leaf sample was placed on a card on top of a wooden board, and the tissue was crushed into the card using a large flat-headed bolt, which was hit hard with a hammer. This technique disrupted cell walls and allowed for transfer of plant DNA to the FTA card, and remaining leaf tissue was removed from the card. Succulent and bromeliad leaves were first scored with a knife to aid DNA transfer into the FTA card. The FTA cards were stored in plastic bags with a Mini-Pax desiccant to prevent contamination. Animal tissue samples were also collected from a range of species in the park (Appendix 1) and DNA from blood, buccal, and fecal samples were stored on FTA Microcards.

DNA from collected leaf samples was extracted using Qiagen DNeasy Plant Mini kits. DNA from wing punches, muscle, heart, and liver tissue was extracted using Qiagen DNeasy Blood and Tissue kits, with the exception of *Artibeus jamaicensis* (bat), which was extracted with a Qiagen DNeasy Plant Mini kit. Modifications to the Qiagen protocol for field use only involved the use of a cold stream water bath to replace the ice bath. Qiagen is a well-known and reliable system of DNA extraction, and was used in this study as a standard against which the effectiveness of FTA cards could be compared.

FTA card punches were 2 mm in diameter. Approximately half of the punch taken contained material, and this paper punch was put in the PCR tube instead of liquid DNA. To clean the plant or animal DNA embedded in FTA cards, punches were washed several times with FTA purification reagent and TE_{0.1} buffer solution as described by Whatman, with several modifications, as follows. Individual washes for punches with plant material were 100 µL, and washes for punches with animal samples were 200 µL. For ferns and pines, the wash with 100 µL TE_{0.1} buffer solution was repeated three times. Punches were dried at room temperature or in the PCR cyclor before they were added to the PCR reaction tube to avoid interaction of the wash buffer with the PCR reagents.

All DNA samples were amplified using PCR. The selected primer was ISSR primer 840 (GA)₈YT. ISSRs are DNA fragments produced by single primer PCRs. The primer is an 18-20 bp anchored microsatellite that is useful on a suite of species [27, 28] and was used to document a positive DNA extraction. Qiagen PCR reaction volume was 25 µL and included Illustra PuReTaq Ready-To-Go PCR Beads, 22.0 µL dH₂O, 2.0 µL primer and 1.0 µL DNA. The FTA PCR only differed in the amount of dH₂O, 23 µL dH₂O, since the paper punch was added to the PCR tube instead of liquid DNA. An Eppendorf Mastercycler was used for amplification with an initial denaturation period of 1.5 min at 94°C; 35 cycles of 40s at 94°C, 45s at 47.2°C, 1.5 min at 72°C and a final 5 min extension at 72°C; 4°C soak. PCR products were visualized on 2.2% Lonza Flash Gel Systems at 175 volts for 12 minutes. Analysis of banding patterns was completed using the Kodak 1D 3.6 program. The efficacy of Qiagen and FTA techniques was analyzed by comparison with PCR success. The two methods of plant DNA preservation/extraction, as well as the seven species sampled, were compared using a log-linear model for the analysis of categorical data in SYSTAT 7.0 [29].

Liquidambar styraciflua study

Leaf tissue samples of ten *L. styraciflua* specimens were collected from each of 3 sites within CNP: 'Base Camp' (15° 29' 50" N, 88° 12' 42" W; altitude 1,546 m), 'Cantiles' (15° 30' 45" N, 88° 14' 23" W; 1,823 m), and 'West' (15° 33' 42" N, 88° 18' 1" W; 498 m) (Fig. 1). Total genomic DNA was isolated using the Qiagen DNeasy Plant Mini Kit. Subsets of leaf tissue samples were used to screen a total of 10 ISSR primers to determine which primers were polymorphic for this species. Three primers produced polymorphic bands (825 [AC]₈T, 840 [GA]₈YT and 855 [AC]₈YT). Analysis of ISSR banding patterns was completed using BioMax

ID image analysis software (Eastman Kodak Company). Band fragment sizes were based on 1-kb ladder size standards, according to the BioMax 1D algorithm. Bands were interpreted as dominant markers and were visually scored as diallelic, regardless of intensity (band presence or absence scored as 1 or 0, respectively). POPGENE 3.12 software [30] was used to measure intraspecific genetic variation among and within *L. styraciflua* populations. Genetic variability statistics, including gene diversity, heterozygosity and percentage of polymorphic loci were calculated as Nei's Gene Diversity and Shannon's index [31]. In addition, to test for a correlation between genetic and geographic distance among the three populations, a Mantel test was conducted using the TFGPA software [32].

Ethics Statement

All samples were collected under permit No 1501-1959-00368 from the Honduran Government. This permit was issued by Santos Edgardo Cruz (AFE COHDEFOR) to Timothy Frederick Coles (Operation Wallacea) and covered all locations of study in Cusuco National Park. All guidelines for care and use were followed as described in the permit.

Results

Testing of molecular techniques and modification of manufacturers' protocols

Transfer of DNA to FTA cards was effective for all species and DNA sources tried, as indicated by PCR success. PCRs run on plant samples from both Qiagen and FTA methods had high success rates (90% and 78%, respectively). To achieve such high success rates, some modifications of the manufacturers' protocols were required, as set out above (see Methods). Neither the species nor the method used (FTA vs. Qiagen) had a significant effect on PCR success rate (Table 1; log-linear model: species: $\chi^2 = 14.41$, $P = 0.2751$; method: $\chi^2 = 2.27$, $P = 0.9435$). For animals, 83% (five successes out of six trials) of FTA samples resulted in successful DNA amplification by PCR. DNA from animal tissue was successfully extracted using Qiagen DNeasy Blood and Tissue kits and was successfully amplified by PCR 100% of the time. In the case of the Jamaican Fruit-Eating Bat, *A. jamaicensis*, DNA from the buccal swab was successfully extracted using a Qiagen DNeasy Plant Mini kit and PCRs were positive for DNA amplification. Positive PCRs resulted from DNA extracts from a total of ten animal species and nine tissue types using both the Qiagen and FTA techniques (Appendix 1). Of these, 90% were positive for PCR analysis using ISSR primer 840.

Liquidambar case study

In total, 90 PCRs produced 573 scorable bands, with sizes ranging from 212-2,967 bp. Genetic diversity was detected among metapopulations of *L. styraciflua*. By all measures, genetic diversity was lowest for the highest-altitude Cantiles samples (altitude c.1,800 m), with Base Camp (c. 1,500 m) only slightly higher and the West site (c. 500 m) exhibiting the greatest variability (Table 2). Mantel test with 1,000 permutations revealed that the genetic divergence of populations was correlated with geographic distance ($r=0.619$, $P=0.0009$).

Table 1 Success rates of PCRs run on plant samples from both Qiagen and FTA methods.

Species (Common Name)	Extraction Method	Number of Trials	Successful trials
<i>Cecropia peltata</i> (Trumpet Tree)	Qiagen	6	5
	FTA	6	5
<i>Lycopodiella cernua</i> (Club Moss)	Qiagen	9	8
	FTA	6	6
<i>Pinus tecunumanii</i> (Red Pine)	Qiagen	8	8
	FTA	10	6
<i>Pinus maximanoi</i> (Grey Pine)	Qiagen	4	3
	FTA	3	3
<i>Tillandsia guatemalensis</i> (Bromeliad)	Qiagen	4	3
	FTA	6	5
<i>Sphaeropteris horrida</i> (Tree Fern)	Qiagen	3	3
	FTA	3	2
<i>Cyathea godmanii</i> (Tree Fern)	Qiagen	7	7
	FTA	11	8
All species	Qiagen	41	37 (90%)
	FTA	45	35 (78%)

Discussion

We demonstrated that molecular genetic analysis can be performed rapidly in remote locations, with basic facilities, volunteer labor, and at relatively low cost. The methods we used were successful for all seven species of plant and all ten species of animal tested, though some modifications to manufacturers' protocols were required. Our *Liquidambar* study, for example, demonstrated one potential use of the methods in a conservation context: identifying areas of highest and lowest intraspecific genetic diversity within a conservation site. More broadly, analysis of molecular markers in the field can help conservation biologists integrate ecology, molecular biology and evolutionary biology to achieve better understanding of a site's biodiversity [1].

A major advantage of being able to conduct molecular analyses rapidly at remote sites is the ability to respond/change experimental design or collection patterns because of molecular genetic data. This is particularly useful for research that is conducted in relatively short field seasons, or where urgent conservation action is required. We argue that the ability to perform molecular genetic work on site,

rapidly and at relatively low cost, will also encourage more DNA-based conservation work. If so, we suggest that DNA obtained from conservation expedition groups be sent to global repositories. For example, ambitious existing programs such as the Tree of Life (aTOL: <http://atol.adsc.edu>) and the Barcode of Life (CBOL: <http://barcoding.si.edu>) already use genetic tools to study global biodiversity [33].

Table 2 Measures of genetic diversity of *Liquidambar styraciflua* from Cusuco National Park, Honduras.

Measure	Base Camp	Cantiles	West
Mean *h	0.276	0.2307	0.3662
Mean *I	0.4094	0.3398	0.5335
Polymorphic loci	31	25	37
% polymorphic loci	75.61%	60.98%	90.24%

*h = Nei's Gene Diversity; *I = Shannon's Index,

Successful implementation of Operation Wallacea's DNA field sampling course illustrates that volunteer tourism can be used not only to collect samples, but also to perform molecular genetic analyses on site. Reliable data production by volunteers has been shown in several recent studies [9,34], and our findings extend this to molecular genetic data production. We found the two alternative techniques, FTA and Qiagen, to have similar reliability in a field setting. The technologies we used are quite affordable and new products are continually being developed. Lonza recently created a gel electrophoresis camera to supplement its FlashGel System. FTA Elute cards and GenTegra™ DNA kits [35] provide possible alternatives to the FTA tissue storage cards used in this study.

North American *Liquidambar* makes an ideal case study, since this tree species has a dynamic biogeographic history and is found commonly in CNP. Recent studies have focused on the phylogeographic history of American and Mexican populations [36-38], and few populations have been sampled from Latin America. Our study is distinctive as it is one of the few population genetic reports on *Liquidambar styraciflua* in Central America. The ISSR analysis of three populations from CNP, Honduras, has demonstrated relatively high levels of genetic diversity and a clear relationship between genetic and geographic distance. Hoey and Parks [36] demonstrated this same pattern using allozymes. Sweetgum is a component of the cloud forest in CNP, and this species has migrated from Mesoamerica to the U.S. [38]. Contemporary cloud forest lineages have a complex evolutionary history, and conservation plans may need to be species specific [39]. More information on population genetic variation in cloud forest species would contribute to a stronger understanding of regional variability, which is important in conservation efforts.

Implications for conservation

We found that the new molecular tools described were successful in collecting, amplifying and analyzing DNA from 17 plant and animal species in basic field conditions. A modest molecular lab is now a viable component of most research stations and can complement existing field studies, which is an asset to research programs where sampling is time sensitive. This is especially appropriate in studying disease spread. A notable example is the spread of chytrid fungus (*Batrachochytrium dendrobatidis*) which has been implicated in the global decline of amphibians [40] and has been documented in Cusuco National Park [41]. It is possible to determine the presence or absence of chytrid in amphibians within days after swabbing in remote locales. These results allow real-time modifications of sampling regimes. Our work documents that field conservation genetics can be done by novice researchers, and greater familiarity with molecular biology may increase genetic sampling in remote regions with high biodiversity.

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Appendix 1. Positive PCR results of DNA extracts from ten animal species and nine tissue types using both the Qiagen and FTA techniques.

Species (Common Name)	DNA Source	DNA Preservation/ Extraction Technique
<i>Sturnira ludovici</i> (Highland Yellow-shouldered Bat)	Wing Punch	Animal Qiagen
<i>Artibeus jamaicensis</i> (Jamaican Fruit-eating Bat)	Buccal Swab	Plant Qiagen
<i>Myotis keaysi</i> (Hairy-Legged Myotis)	Wing Punch	Animal Qiagen
	Heart	Animal Qiagen
	Liver	Animal Qiagen
	Muscle	Animal Qiagen
<i>Carollia brevicauda</i> (Silky Short-tailed Bat)	Wing Punch	Animal Qiagen
	Heart	Animal Qiagen
	Liver	Animal Qiagen
	Muscle	Animal Qiagen
<i>Chrysina karschii</i> (Jewel Scarab Beetle)	Leg	Animal Qiagen
<i>Heteromys desmarestianus</i> (Spiny Pocket Mouse)	Blood	FTA
	Ear	Animal Qiagen
<i>Peromyscus mexicanus</i> (Mexican Deer Mouse)	Blood	FTA
	Buccal Swab	Animal Qiagen
<i>Ninia espinali</i> (Espinal's Coffee Snake)	Buccal Swab	FTA
<i>Sceloporus malachiticus</i> (Emerald Swift Lizard)	Faecal Swab	FTA
	Buccal Swab	Animal Qiagen
<i>Scotinomys teguina</i> (Alston's Brown Mouse)	Buccal Swab	FTA