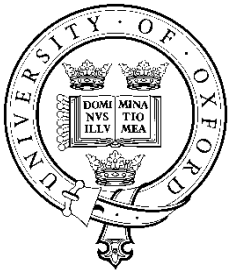

UNIVERSITY OF OXFORD

FHS IN BIOLOGICAL SCIENCES

UNDERGRADUATE PROJECT REPORT

PROJECT DISSERTATION SUBMITTED AS PART OF THE FINALS EXAMINATION IN THE HONOURS SCHOOL IN BIOLOGICAL SCIENCES

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COVER SHEET PAGE 1

Project Supervisor	**ROBERT PUSCHENDORF (PLYMOUTH UNIVERSITY)** **(ALEX ROGERS – INTERNAL SUPERVISOR)**
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TITLE:

Genetic Diversity in the Last Populations of *Craugastor ranoides*

Abstract

Across the globe amphibian populations are declining due to chytrid disease, climate change and habitat loss. However, the rate of this decline in terms of the numbers of species lost is difficult to determine, due to the cryptic diversity contained in many amphibian lineages. DNA barcoding using 16S and CO1 gene sequences provides a rapid assessment of cryptic diversity, and the relationships between individuals in sympatric populations.

Some amphibian species persist in lowland areas, known as climatic refugia, which protect them from a high prevalence of *Batrachochytrium dendrobatidis*. One such species, which persists only in the Santa Elena Peninsula, Costa Rica, is the critically endangered *Craugastor ranoides*.

Study Conclusions:

1. The persistence of *C.ranoides* in three streams in the Murcielago Sector of the Area de Conservation Guanacaste, and in one tributary of the Rio Portero Grande, the Quebrada la Danta was confirmed, and one additional population of *C.ranoides* was recorded for the first time in the Rio Nisperal, in the Naranjo Valley.
2. No *C.ranoides* individuals were found in the Rio la Calera, another tributary of the Rio Portero Grande, where populations had previously been recorded.
3. Analysis of tissues collected from the five populations sampled, using isolated 16S and CO1 genes, revealed that the individuals from five sites constituted the same species, not differing significantly from previous *C.ranoides* individuals sampled.

Thus, proposed relocations from these relictual populations, to some of the areas where they no longer persist, such as Volcan Cacao, would be recommended, based on this analysis.

Introduction

Amphibian Declines

Since the 1950s, when major amphibian declines were first reported (Alford et al. 2001) the combination of habitat loss, climate change, overexploitation, invasive species (Collins and Storfer 2003) and particularly, the infection of amphibian populations with the fungal disease chytridiomycosis, has led to the categorisation of amphibians as the most threatened group of vertebrates (Stuart et al. 2004). These population declines are not only of concern due to their global nature (Lips et al. 2003), but, in addition, their occurrence in protected and relatively untouched habitats presents serious challenges in terms of the formulation of conservation plans In 2015, the IUCN classifies 41% (6,414 species) (IUCN 2014, IUCN 2014 2014) of species from the class *Amphibia* as threatened; classified as critically endangered, endangered, or vulnerable. (IUCN 2014 2014)

Latin America in particular contains an incredible diversity of amphibian species; indeed, half the global complement of amphibian diversity is housed in this region.(Duellman 1999, Young et al. 2001). As a result, it has born its share of the burden of disease and habitat loss driven declines. For example, in Costa Rica alone 2 frog species have been classified as extinct, and 10 of the 19 critically endangered species which inhabit this country have not been sighted since the mid-1980s to early 1990s, when declines were first reported in this region. (García-Rodríguez et al. 2012)

Studies carried out on amphibians which attempt to quantify the characteristics of species or regions which might be more vulnerable to population decay, potentially assisting in conservation planning, (Lips et al. 2003) are hampered by not only the continuing decline of amphibian populations (Crawford et al. 2007), but also the large percentage of amphibian species which remain undescribed or undiscovered. Programmes which attempt to conserve

these threatened species are designed based upon species lists, which can be corrupted by incomplete taxonomy (Crawford et al. 2013), and thus, characterisation and genetic exploration of amphibian diversity is key to its conservation. In 2001, it was reported that 5-10% of the Central American amphibian fauna could remain undescribed, clearly a barrier to efficient implementation of conservation and management strategies.(Young et al. 2001) Indeed, in one analysis of the lineage diversity of an amphibian community in Panama, before and after a mass-mortality event, it was discovered that of the 30 species lost, 5 remained undescribed. (Crawford et al. 2010)

Climatic Refuge Theory

Despite the continuing decline of many amphibian populations, some species which were previously thought to be extinct have been rediscovered, such as *Atelopus varius, Incilius holdridgei* and *Lithobates vibicarius* in Costa Rica, one of the best tropical study systems for such declines. (Abarca et al. 2010, García-Rodríguez et al. 2012) These rediscoveries have occurred in areas of previous chytrid prevalence, and low annual precipitation. (García-Rodríguez et al. 2012, González-Maya et al. 2013) One of the most severely affected groups is the direct developing *rugulosus* group of *Eleutherodactylus* frogs. (Campbell and Savage 2000)

C.ranoides is one of the species in this group, which was re-discovered in the Santa Elena Peninsula, perhaps the seasonally driest site in Costa Rica. (Janzen 1998, Zumbado-Ulate et al. 2014) These rediscoveries present the possibility that other species previously thought to be extinct could in fact persist in dry forest regions at the periphery of their ranges, where *Batrachochytrium dendrobatidis*, the fungus causing chytrid disease is limited in its prevalence due to high temperatures and low precipitation. (Puschendorf et al. 2013). Areas which represent the edge of the climatic profile inhabited by *Batrachochytrium dendrobatidis*

have been proposed to provide refuge from disease to even those species which are highly susceptible to infection, such as the *Craugastor rugulosus* species series.

Craugastor ranoides Morphology and Phylogeny

The species *C.ranoides* is part of the *Craugastoridae* family (Solis, 2014), and the *Craugastor rugulosus* species group, formed of 34 riparian species with direct development. (Campbell and Savage 2000) This species group is threatened at all altitudes of its distribution (Zumbado-Ulate et al. 2011), and has been severely affected by the decline of amphibian populations in Central America.(Campbell and Savage 2000) Currently five species are classed as critically endangered, and two are classed as data-deficient.

Many deeper branches in the phylogeny of the *Craugastoridae* family remain unresolved, however, strong support has been found for the separation of *Craugastor ranoides* from its closest relative, *Craugastor rugulosus*, and its separation from *Craugastor obesus* and *Craugastor punctariolus*. (Alexander Pyron and Wiens 2011) The separation of *C.ranoides* from *Craugastor fleishmanni*, a species from the rugulosus group which overlaps with *C.ranoides* in its range on the pacific coast of Costa Rica requires confirmation from genetic data. (Campbell and Savage 2000) Currently *C.ranoides* can be distinguished by the marking of their dark posterior thigh with light spots, unique to this species in comparison to other *ruglosus* species in Costa Rica. (Savage 2002) Phylogenetic trees constructed in this study display the need to resolve the taxonomy of this group in more detail, due to the genetic difference between outgroup sequences, a problem which is amplified by the preservation of many ancestral species from this family in formalin, which cannot be easily analysed.

Distribution Information to Date

The critically endangered status of *C.ranoides* was invoked by the IUCN red list in 2008, due to the severe population declines it has sustained, thought to be more than a decline of 80%

over the last three generations. (Solis 2014) After surveys in the majority of *C.ranoides* range in Costa Rica presented no sightings from 1986 to the present, including sites such as Volcan Cacao where it was previously common, the distribution of *C.ranoides* was concluded to be restricted to the Santa Elena Peninsula.

One outstanding characteristic of the Santa Elena Peninsula is the continuous flow of springs and deep pools at major rivers throughout the dry season, from December to April, key to the persistence of riparian species. Previous studies have identified populations of C.ranoides in the Rio Murcielago sector on the Santa Elena Peninsula, and in two tributaries of the Rio Portero Grande; the Quebrada la Danta and the Rio La Calera. (Zumbado-Ulate et al. 2007) Between 2002 and 2011, when these three rivers were recorded to harbour populations of *C.ranoides*, the range of *C.ranoides* has significantly decreased in size, disappearing from the Caribbean slope, the southern Pacific and the Central Valley, a decline at all elevations in Costa Rica. (Puschendorf et al. 2009, Zumbado-Ulate et al. 2011) In order that these last persisting populations be maintained, it is essential to characterise and understand their distribution, with the addition of genetic data. In this study, we seek to confirm the continued existence of these populations, and also explore an additional site in the Naranjo valley.

Genetic Monitoring and Analysis of Amphibian Populations

In order to consider the movements and origin of the populations of *C.ranoides* persisting in Costa Rica, genetic data can be used as an invaluable key to the history of the species. When attempting to design amphibian conservation programmes, particularly with regard to translocations, and ex situ breeding, examining genetic data should be an integral part of the planning process. (Crawford et al. 2013) In the case of amphibians, where cryptic diversity within populations may be the rule rather than the exception (Vences et al. 2005b) the identification of cryptic species through DNA barcoding can be key to the success of

conservation strategies, though information from ecology, morphometics and acoustics should also be incorporated into the species determination process. (Crawford et al. 2013) This is due to the masking effects of convergence and parallelism in genetic data, which can obstruct the discovery of diversity if other factors are not taken into account. (Vences et al. 2005b)

Comparative phylogeny can also provide information on the historical processes which have moulded a species' distribution, through the level of phylogeographic structure of species and populations. (Crawford et al. 2007) In these cases, a strong phylogeographic structure can point towards environmental barriers, such as mountainous and dry areas, having a preventative impact on gene flow, and thus, potentially creating cryptic diversity. (Crawford et al. 2007)

Genetic data could also provide an insight into the source of differing immune responses to infection with Bd (Zumbado-Ulate et al. 2014), as the absence or presence of genetic divergence between persisting and declining populations may indicate whether environmental or genetic factors are causing these differences in reaction to disease. In one such case, the phylogeny of *C.fitzingeri* suggests a high level of gene flow across its distribution in the Golfo Dulce region (Crawford et al. 2007), and thus, differences in population persistence with Bd can be more strongly attributed to environmental constraints on fungal growth, rather than differences in host resistance. (Zumbado-Ulate et al. 2014)

DNA Barcoding of Amphibians

In order to collect this data, and quantify lineage diversity in the absence of a complete taxonomy, DNA barcoding, the identification of species by short sequences of DNA, has been suggested as an effective first assay for hidden cryptic diversity in amphibians. (Vences et al. 2005b) This project has become a well-funded and worldwide operation since its

inception in 2003 (Hebert et al. 2003, Taylor and Harris 2012) due to its potential to create valuable links between museum collections and field specimens, and allow the discovery of new species. (Smith et al. 2008) In order to rapidly assess the genetic diversity of sympatric populations on a single site based on barcode data, many studies have used clustering algorithms based on genetic distance in order to group species into Operational Taxonomic Units. (Paz and Crawford 2012) This method relies on the assumption that intraspecific divergences will be less than interspecific divergences, and thus, the idea of a divergence threshold, or barcoding gap. (Meyer and Paulay 2005)

One of the fragments of DNA used in this study, a 5' fragment of the mitochondrial gene for cytochrome oxidase subunit I (COI), has been proposed as a universal marker for animal species, due to its success at species delimitation in arthropods and birds. (Vences et al. 2005a) However, due to some studies finding the CO1 DNA barcode primer regions variable in amphibians, in general, many more amphibian taxa have been sequenced for 16S rRNA DNA. (Vences et al. 2012) The mitochondrial 16S rRNA gene is a highly conserved mitochondrial marker; however, mutations are common in some variable regions. Thus far, no universal primers have found success in CO1 amplification across all amphibian taxa, which can lead to the necessity of adjusting the PCR strategy depending on the specific research question. In contrast, the mitochondrial DNA 16S ribosomal rRNA gene is a well conserved mtDNA gene sequence in amphibians (Maya-Soriano et al. 2012), and thus, primers can be designed specifically to encompass the species delimitation across many clades.

A combination of genes, along with data from morphology, is always more reliable in assaying diversity, as the genetic structure of amphibian genes might not allow identification through DNA barcodes based on cytochrome oxidase subunit 1 sequences in all cases. Further problems arise in the species delimitation of amphibians due to their tendency to

contain deep conspecific lineages, which could lead to incorrect species identification when reference databases are incomplete. (Vences et al. 2012)

Thus, in this study, in order to determine whether individuals across sites, separated by mountainous divides, constitute the same species, both mitochondrial 16S and CO1 genes are used, along with data on morphology and ecology of the populations studied. In order to test the hypothesis that these allopatric populations are genetically indistinct, a distance based approach to species delimitation was used to analyse the data. The sequences collected were also compared to barcode region sequences of *C.ranoides* from Genbank, in order to determine if correct identification of species had occurred in previous studies.

Methods

Field Work

Figure 1 - Sites where C.ranoides populations have been recorded

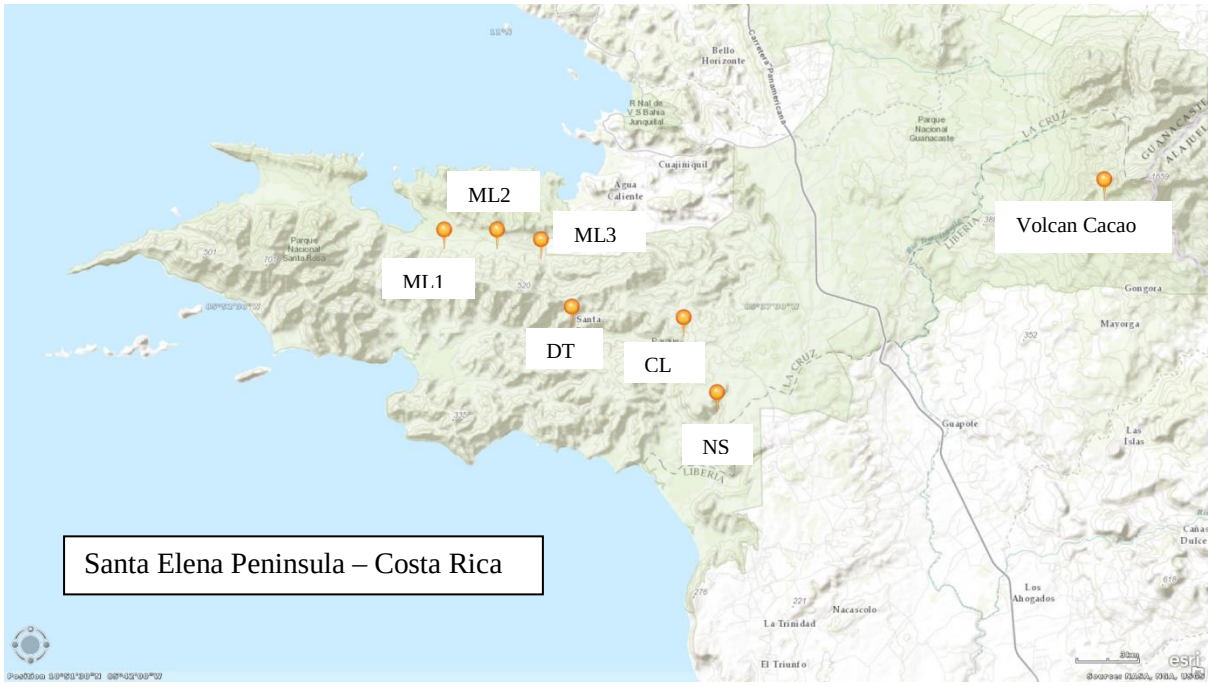

Table 1- Coordinates of populations of C.ranoides (extant and extinct)

Site	Latitude	Longitude
Murcielago Creek 1 (ML1)	10.90134	-85.77522
Murcielago Creek 2 (ML2)	10.90135	-85.75068
Murcielago Creek 3 (ML3)	10.89691	-85.73029
Quebrada la Danta	10.86623	-85.716
Rio la Calera	10.86139	-85.66409
Rio Nisperal	10.8273	-85.6486
Volcan Cacao (Exinct)	10.924208	-85.468837

Murcielago Sector

In the Murcielago sector of the Area de Conservation Guanacaste, previous studies recorded populations of *C.ranoides* in three creeks. (Sasa and Solórzano 1995, Zumbado-Ulate et al. 2007, Zumbado-Ulate et al. 2014) Sampling was started from the point where the stream emerged from underground in all three cases. In the case of the Murcielago sector, sampling seemed to be more successful in still conditions, and in the early evening.

Table 2 - Details of sampling and conditions at sites in the Murcielago sector

Site	Date(s) Sampled	Time	Conditions	Coordinates	Creek details
ML1	27/6/14	18:35 – 20:56	Still, cloudy, temperature 24°C	10.90134 N, 85.77522W	Alt – 22m asl, low water level, large boulders, and lack of vegetation
ML2	27/6/14	21:32 – 22:29	Still, cloudy, temperature 24°C	10.90135N, 85.75068W	Alt – 13m asl, larger creek with more vegetation
ML3	27/6/14	11:02-11:45	Still, cloudy, temperature 24°C	10.89691N, 85.73029W	Alt – 9.7m als, largest creek with deep pools and steeper rocks surrounding, including the 'generals pool', slow flowing, and vegetation on either side. Larger animals maybe inhabit upstream area, croc seen.
ML3 (second sampling)	29/6/14	19:00-20:18	Wind – 4km/hr, humidity 70%, temp 28°C	10.89691N, 85.73029W	No rain during the day, and river seen to be extremely low for this time of year

Table 2 - Details of sampling and conditions at sites in the Murcielago sector The third creek to be sampled in the Murcielago sector was sampled three times, due to disappointing results on the first date of sampling, and populations being reported there in previous years. ML3 was noted to have an extremely low river level for this time of year, and an earlier time of evening for sampling was found to be more productive.

Tributaries of the Rio Portero Grande

As a result of previous reports of populations of *C.ranoides* in two tributaries of the Rio Portero Grande, the Quebrada La Danta and the Rio La Calera (Zumbado-Ulate et al. 2007), these rivers were searched for *C,ranoides* on the 28/6/14. These rivers are contained in an area of the Area de Conservation Guanacaste which has extremely restricted access, is relatively untouched by humans, and thus represents a good place to study population declines in pristine habitats.

Table 3 - Sampling and conditions at tributaries of the Rio Portero Grande

Site	Date(s) Sampled	Time	Conditions	Coordinates	Creek details
DT	28/6/14	18:51-20:45	Still, 23.2°C, 46% humidity	10.86623N, 85.71600W	Alt- 111.6m asl slow, continuously flowing, large boulders and waterfall with area of pH 11/12 due to tributary upstream (sampled above and below this)
CL	28/6/14	21:30-22:20	Still, 23.2°C, 46% humidity	10.86139N, 85.66409W	Alt – 30m, stream extremely dry, tapir faeces found and algal growth in water

Table 3 - Sampling and conditions at tributaries of the Rio Portero Grande - Both rivers were noted to be extremely low for the wet season and in the Rio Calera tapir faeces were seen alongside the stream, along with a large amount of algal growth in the water. This may have altered the stream conditions, and rendered them unsuitable for C.ranoides,in comparison to sampling in 2007 (Zumbado-Ulate et al. 2007) when individuals were recorded at this site.

Nisperal River

On finding flowing water, which is required by *C.ranoides*, we sampled upstream from this position on the Nisperal River, in the nearby Naranjo valley, where *C.ranoides* has not previously been found. 18 *C.ranoides* individuals were sighted, an exciting find on this side of the mountainous divide between the Murcielago Sector, and the Rio Portero Grande and Rio Nisperal. 5 individuals were sampled.

Table 4 - Sampling and conditions on the Nisparan River

Site	Date(s) Sampled	Time	Conditions	Coordinates	Creek details
NS	1/7/14	18:48-20:18	Wind speed – 12km/hr, 28^{o}C, humidity 74%	10.8273N, 85.6486W	Alt – 103.2m asl, river dry downstream, but slow flowing and continuous upstream, waterfall with large boulders, and little vegetation

Table 4 - Sampling and conditions on the Nisparan River - This sampling site was only visited on one occasion, due to reports that it was dry, however, 18 individuals were sighted at the flowing water found.

Species Identification

C.ranoides individuals were identified by their distinctive hind leg markings, the slight characteristic webbing on their feet and more rotund bodies and heads in comparison to the common frog *C.fitzingeri,* a species which also inhabits this area. (Savage 2002)

Figure 2 - Example of C.ranoides female morphology

Figure 2 - Example of C.ranoides female morphology - The photos below show a large C.ranoides female, sighted on the Quebrada la Danta on the 28/6/14, showing characteristic patterns on thigh and venter colouration.

Gender differentiation

In order to determine the sex of the individuals captured, both size and morphology were examined, due to the larger size of adult females (40-74mm in length) in comparison to adult males (26-45mm in length) (Savage 2002) Males also have a larger tympanum diameter than females, due to the production of advertisement calls, which was also used to determine sex. In the case of some of the juvenile individuals sampled, sex could not be determined due to sexual dimorphism not yet being developed.

Sampling effort

GPS data tracked the distance travelled during active sampling along the named creeks chosen, recording a point every 10m. Sampling was only ever conducted after sunset, as late dusk proved the most likely time of day to observe C.*ranoides*. Sampling was always conducted at a steady pace, and usually involved walking upstream.

Total sampling time (sampling effort) was calculated using the equation below;

$$no.of\ samplers(time\ until\ first\ indiv.) + (no.of\ samplers - 2)(remaining\ sampling\ time) = total\ sampling\ time(effort)$$

Sampling details for each night of field work are contained in Table 15- Details of Sampling by Date **in the supplementary information.**

Data Collection

When a *C.ranoides* individual was identified, its position was marked with the GPS, and the time, conditions and microhabitat recorded.

Handling – in order to minimise stress on the frogs, individuals were firmly grasped between the thumb and index finger on the hind legs, letting the frog rest its body on the back of the hand. No insect repellent containing DEET was worn. Individuals were always returned to the site of capture due to the territorial nature of this species.

Figure 3 - Swabbing Protocol

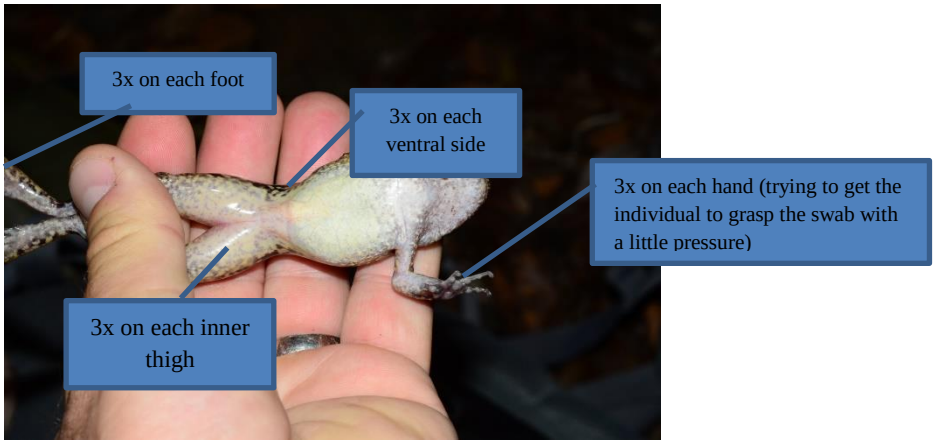

Figure 3 - Swabbing Protocol - in order to detect the presence or absence of any potential pathogens, particularly Bd, which causes worldwide amphibian declines, swabbing was always carried out with a sterile swab. Frogs were laid ventral side up in the palm and swabbed in a consistent methodical manner

Table 5 - Morphometric Measurement Methods

Measurement	Method
Snout-vent length (mm)	Measured whilst holding the individual sampled dorsal side up, and using callipers.
Leg length (mm)	The length of the femur was measured, using callipers.
Head width (mm)	Measured using distance between the eyes of the individual frog, using callipers.
Weight (g)	Measured by difference in sealable plastic bags using a spring balance.

Table 5 - Morphometric Measurement Methods - when recording the above measurements, the researcher was kept consistent reducing sampler error, which can particularly affect spring balance data.

Toe clipping and tissue storage. As permits (ACG permit number - ACG-PI-036-2014, CONAGEBIO Permit number - R-036-2013-OT-CONAGEBIO) only allow Robert Puschendorf to toe clip the frogs, he took samples in order to obtain tissues, using a pair of small curved scissors. One toe end per individual was cut off at the edge of the cartilage and placed in a tube of ethanol. In some cases, as with females carrying eggs, toe clips were not taken in order to reduce stress, as these individuals are highly valuable in terms of conservation.

Lab Work

DNA extraction

In order to extract the DNA from the toe clip tissues collected in the field, a standard ammonium acetate extraction protocol was used.

Nanodrop samples

A NanoDrop analysis was used to test the amount of DNA contained and the purity of the 24 extractions. The spectrophotometer was set to absorb at 260nm. (NanoDrop Technologies inc. 2007) As none of the concentrations dropped below 2ng/ml, the samples were run through PCR (CO1 and 16S).

16S + C01 PCR

The original recipes followed for the PCR reactions can be found in the supplementary information Table 16 and Table 17. In order to increase the success of the amplification of the CO1 gene some alternative recipes were created. In the initial PCR reactions, a 20uL reaction volume was used for each sample, and a master-mix of all reaction ingredients except gDNA was made for 25 reaction volumes (24 samples + 1 error). The 10x buffer (containing 15mM $MgCl_2$), dNTPs (dNTP mix 10mM), Mg2+ (25mM $MgCl_2$) and Taq DNA polymerase (5 units/uL) used originated from a Qiagen Taq PCR core kit. (QIAGEN Group 2011)

Primers Used

Table 6 - CO1 & 16S Primers

Gene	Primer sequence (F)	Primer sequence (R)
CO1	F 5'– TAA ACT TCA GGG TGA CCA AAR AAY CA –3'	R 5'– GGT CAA CAA ATC ATA AAG AYA TYG G –3'
16S	F 5'– CCG GTC TGA ACT CAG ATC ACG T –3'	R 5'– CGC CTG TTT ATC AAA AAC AT –3'

The reaction master-mixes contained 2uL of DNA sample per 18uL of master-mix for each gene region. Reaction mixes were placed in the pre-programmed thermal cycler (*Techne Prime Thermal Cycler*) for 34 cycles, following the detailed times and temperatures in the supplementary Table 21.

After disappointing CO1 PCR results with the inclusion of BSA (*bovine serum albumin*), reported in several papers to increase the success of amplification of CO1 through a decrease in the effects of PCR inhibitors, (Che et al. 2012, Smithsonian Institution,) BSA was replaced with ddH_2O, in order to isolate the inhibiting factor in the CO1 PCR. 10uL of PCR reaction volume was used. On analysis through gel electrophoresis this recipe produced correct amplification of CO1 from at least one sample per site.

The CO1 PCR was repeated using three experimental recipes which included BSA, excluded BSA and an included an increased percentage of BSA per reaction mixture, using the same percentage volume per reaction mixture as used by Che et al., 4.8% volume per reaction mixture (0.48uL BSA per 10uL). (Che et al. 2012)

The experimental PCR recipes can be found in the supplementary information Table 18, Table 19 and Table 20

Gel electrophoresis

Both 1% and 2% agar gels were trialled, due to the use of 2% gels in some previous amphibian studies (Smith et al. 2008), with the 1% gel being found to be more appropriate for the experiment. Gels were run for approximately 20 minutes using a TAE buffer (Philips 2015) (Tris-acetatE-EDTA) x1M on 100V (90mA), supplied by the Amersham pharmacia biotech powerpack.

UV Exposure and Visualisation

Gel electrophoresis runs were exposed and visualised on the ImageQuant LAS4000. Settings used were; Exposure type- Precision, Exposure time – 12 seconds, Resolution- Standard, Temperature- -26^{o}C, Fluorescence- SYBR Green, Tray height-3.

Gene Sequencing

Both the 16S and CO1 PCR products were sent to MACROGEN Inc. for sequencing. All non-BSA samples were sent (from both 16S and CO1 products), along with the re-runs of CO1 non-BSA and 8 samples which used increased concentration of BSA.

Bioinformatics

The *Geneious* bioinformatics software was used in order to assemble forward and reverse reads from the chromatographs of the 16S rRNA and CO1 rRNA. (*Geneious Version 8.1*)(http://www.geneious.com, (Kearse et al. 2012) The bidirectional sequence data was first trimmed at the 3' and 5' ends where low quality reads were present, at a probability limit of 0.01. The heterozygous bases were then identified using *Geneious* heterozygote finder (peak similarity – 50%). After which, forward and reverse sequences were assembled into contigs for each individual, most by de novo assembly, and those which contained indels, or were of a lower quality, were assembled to reference sequences.

Any heterozygous alleles were checked manually, along with any disagreements in calls between the forward and reverse sequences in order to create final consensus sequences for each individual sampled. Table 7 and Table 8 below show the number of sequences successfully assembled into consensus sequences from each site, and used to create the phylogenetic trees of CO1 and 16S genetic difference between the sites.

Table 7 - Sequences used by Population (CO1)

CO1 Sequences

Site	No. of Sequences	Short sequence numbers
Murcielago Sector 1	3	1, 4, 5
Murcielago Sector 2	1	9
Murcielago Sector 3	3	10, 13, 16
Quebrada la Danta	1	11
Nisperal River	3	18, 20, 21
Total Sequences	11	

Table 8 - Sequences used by Population (16S)

16S Sequences

Site	No. of Sequences	Short sequence numbers
Murcielago Sector 1	6	1, 2, 3, 4, 5, 6
Murcielago Sector 2	3	7, 8, 9
Murcielago Sector 3	5	10, 13, 14, 15, 16
Quebrada la Danta	2	11, 12
Nisperal River	5	17, 18, 19, 20, 21
Total Sequences	21	

Outgroups were first pairwise aligned on *Geneious* to one of the 16S or CO1 consensus sequences using a global alignment with free end gaps, (gap open penalty – 12, gap extend penalty – 0). A consensus alignment was then created of the consensus sequences with the Geneious alignment tool, using a global alignment with free end gaps, and a cost matrix of 93% similarity (5.0/-9.026168). The same gap penalties were used as previously.

Through geneious, the MrBayes plugin was used in order to create a Baseian inference tree for both the COI and 16S sequences (Substitution model – HK85, chain length – 4,000,000, burn in length – 100,000, and subsampling frequency – 1000) using sequences from the *Craugastor rugulosus* species group in the trees, and using *Craugastor fitzingeri* as an outgroup. (Huelsenbeck and Ronquist 2001) The raw output trees were exported into figtree in order to be rooted (Rambaut 2014).

In order to asses species groups in the CO1 and 16S trees generated, a species delimitation analysis was conducted, using the species delimitation plugin on Geneious, which calculates the probability of reciprocal monophyly against the null model of random coalescence. (Masters et al. 2011) The main clades on the tree were selected as focal groups, and the ratio of intraspecific genetic distances to interspecific genetic distances calculated and analysed.

Results

Field data Collected

Table 9 - C.ranoides sighted with morphological data – (Below) - Date sighted, site found, time of evening found, sex of frog, Snout vent length, Leg length, Head width, weight of frog and remarks on the conditions of sampling, or the condition of the individuals. In five of the six sites visited, populations of Craugastor ranoides were found. No C.ranoides were found on sampling at the Rio la Calera (10.86139N, 85.66409W), where previous studies had found breeding populations in 2007. (Zumbado-Ulate et al. 2007)

Table 9 - C.ranoides sighted with morphological data

Species	Date	Site	Time	Way-point	DNA #	Sex (F/M)	Sn-Vent (mm)	Leg Length (mm)	Head Width (mm)	Weight of bag (g)	Weight of b + frog (g)	frog weight (g)	Remarks
C.ranoides	27/06/2014	ML1	18:45	115	RP56	unable to sex	31.70		9.10	5.00	8.50	3.50	Juvenille, leaf litter 2m from water (20 mins no-one sampling)
C.ranoides	27/06/2014	ML1	19:15	116	RP57	F	46.90	27.00	13.30	6.00	18.00	12.00	Adult, leaf litter under tree 1m from water
C.ranoides	27/06/2014	ML1	19:45	118	RP58	M	29.50	13.90	11.20	5.50	9.10	3.60	Adult, leaf litter, interesting red markings on back
C.ranoides	27/06/2014	ML1	20:13	119	RP59	F	54.00	26.30	14.10	5.00	27.50	22.50	Adult
C.ranoides	27/06/2014	ML1	20:15	119	RP60	M	30.20	16.10	10.10	7.30	11.20	3.90	Adult
C.ranoides	27/06/2014	ML1	21:37	120	RP61	F	48.20	22.20	16.10	5.00	25.10	20.10	Adult
C.ranoides	27/06/2014	ML2	21:56	121	RP62	M	27.80	16.10	10.10	3.00	7.00	4.00	Juvenile, next to waters edge of rocky pool
C.ranoides	27/06/2014	ML2	22:00	122	RP63	M	34.50	16.20	10.10	2.50	9.10	6.60	Juvenile, rock in middle of stream
C.ranoides	27/06/2014	ML2	22:22	123	RP64	F	42.60	24.20	13.30	2.10	17.20	15.10	Adult
C.ranoides	27/06/2014	ML3	23:40	124	RP65	M	32.70	18.10	11.20	5.00	11.20	6.20	Adult, under rock in middle of stream
C.ranoides	28/06/2014	DT	18:55	126	RP66	unable to sex	25.80	11.40	9.80	2.50	5.10	2.60	Juvenile, on rock at edge of stream, upstream of waterfall and alkaline tributary
C.ranoides	28/06/2014	DT	19:10	127	RP67	F	50.30	24.10	15.30	5.00	22.00	17.00	Adult, In shallow water upstream of waterfall and high pH area, potentially a hybrid
C.ranoides	28/06/2014	DT	19:18	128	RP68	unable to sex	22.00	14.10	7.60	5.00	5.50	0.50	Juvenile, 10cm from stream, upstream of waterfall and alkaline tributary
C.ranoides	28/06/2014	DT	19:58	129	RP69	F	54.20	28.60	17.80	5.00	26.50	21.50	Large female, not toe clipped, 10m from stream downstream of waterfall
C.ranoides	29/06/2014	ML3	19:15	130	RP70	unable to sex	35.10	17.50	9.70	2.00	7.50	5.50	Juvenile, Under rock at edge of stream
C.ranoides	29/06/2014	ML3	19:18	130	RP71	unable to sex	32.40	16.40	10.80	5.00	10.50	5.50	Juvenile, found on small tree branch over hanging stream
C.ranoides	29/06/2014	ML3	19:40	131	RP72	F	47.50	23.60	11.40	2.00	18.00	16.00	young female
C.ranoides	29/06/2014	ML3	19:45	132	RP73	F	44.10	20.80	14.20	2.50	16.50	14.00	
C.ranoides	29/06/2014	ML3	20:13	135	RP74	F	ESCAPED						Large adult female, escaped before could measure. Probably at weight off balance scale. Found under rock at edge of stream.
C.ranoides	29/06/2014	ML3	20:15	133	RP75	F	51.40	28.80	17.90	5.00	>30	>25	Large adult female carrying eggs, heaviest so far. Found on rock at edge of stream
	01/07/2014	NS	19:42	136	RP76	F	42.50	25.30	14.70	2.00	17.00	15.00	Adult female found on top of riffle
	01/07/2014	NS	19:43	136	RP77	F	45.70	19.60	13.10	5.00	21.50	16.50	Adult female on top of riffle
	01/07/2014	NS	19:44	136	RP78	JF?	34.20	16.20	10.70	2.00	7.00	5.00	Juvenile female? On top of riffle
	01/07/2014	NS	20:05	137	RP79	F	48.50	24.70	14.20	2.50	21.00	18.50	Adult female on dry rock in bigger waterfall
	01/07/2014	NS	20:06	137	RP80	JF?	32.40	15.80	10.80	6.50	11.50	5.00	Juvenile female? In bigger waterfall. Damp patch
AVERAGES						9J, 13F, 3M	39.34	20.30	12.36			10.44	

As in previous studies, three creeks in the Murcielago sector housed breeding populations, which were seen to be in good health by the presence of egg carrying females. (Sasa and Solórzano 1995, Zumbado-Ulate et al. 2007, Zumbado-Ulate et al. 2014). Both adult and juvenile individuals were found, each being found on rocks adjacent to the stream, on tree branches or in leaf litter.

In the Quebrada la Danta one sampling session revealed a population of *C.ranoides* which again contained breeding females. Individuals were found both above and below the high pH tributary of this stream.

Despite extremely dry conditions in the lower regions of the Rio Nisperal, which runs through the Naranjo valley, flowing water was found on this watercourse and 18 individuals were sighted over sampling for 90 minutes. Due to time constraints, only five individuals were measured and toe clipped, however, the presence of this newly recorded population is extremely encouraging for the conservation of this species.

Morphological data by sex

Table 10 - Adult morphology and site found

DNA Number	Sex	SVL (mm)	Head Width (mm)	HW % of SVL	Inside reported % range? (F = 37-42%, M = 35-41%)	Leg Length (mm)	Weight (g)	Site Code
RP57	Female	46.9	13.3	28.36	no	27.0	12.0	ML1
RP59	Female	54.0	14.1	26.11	no	26.3	22.5	ML1
RP61	Female	48.2	16.1	33.40	no	22.2	20.1	ML1
RP64	Female	42.6	13.3	31.22	no	24.2	15.1	ML2
RP67	Female	50.3	15.3	30.42	no	24.1	17.0	DT
RP69	Female	54.2	17.8	32.84	no	28.6	21.5	DT
RP72	Female	47.5	11.4	24.00	no	23.6	16.0	ML3
RP73	Female	44.1	14.2	32.20	no	20.8	14.0	ML3
RP75	Female	51.4	17.9	34.82	no	28.8	25.0	ML3
RP76	Female	42.5	14.7	34.59	no	25.3	15.0	NS
RP77	Female	45.7	13.1	28.67	no	19.6	16.5	NS
RP79	Female	48.5	14.2	29.28	no	24.7	18.5	NS
RP58	Male	29.5	11.2	37.97	yes	13.9	3.6	ML1
RP60	Male	30.2	10.1	33.44	no	16.1	3.9	ML1
RP65	Male	32.7	11.2	34.25	no	18.1	6.2	ML3

Table 10 - Adult morphology and sit – Details of the adult Snout-Vent length (mm), Leg length (mm), head width (mm), weight (g) and site found. Along with a comparison of this data to previously reported head widths as a percentage of Snout Vent Length. This data can be used to verify taxonomy using morphometric data from previous studies.

Females measured had a SVL (Snout-Vent length) of 42.6-54.2 mm ($\bar{x}$ – 47.992), which is smaller than the mean described by Savage et al. 2000 for the *rugulosus* species series, and at the lower range of body length described in 2002 (40-74mm) (Savage 2002). None of the individuals measured conformed to the reported head width of 37-42% of SVL for females of this species ($\bar{x}$ – 30.23% of SVL). (Campbell and Savage 2000) As genetic results showed that these individuals were indeed from the species *C.ranoides*, this could indicate that

females are reaching smaller sizes in these relictual populations in comparison to 15 years ago.

Males measured had a SVL of between 32.7-29.5 mm ($\bar{x}$ – 30.8mm), falling within the reported 28.9-45mm SVL length reported in 2000 for the *rugulosus* species series, and within the specific species range of 26-42mm reported in 2002. (Savage 2002) The mean HW as a percentage of SVL also conformed to the 35-41% ($\bar{x} - 35.23\%$) reported in 2000 for *C.ranoides*, though it should be noted that only one of the individuals found actually fell in this range, and with the low numbers of male frogs found, conclusions cannot be drawn from these results. (Campbell and Savage 2000)

Despite the small size of the female individuals measured, sexual dimorphism was still observed in mean SVL, leg length, head width and weight of adult individuals, as reported in previous studies. (Campbell and Savage 2000, Savage 2002, Zumbado-Ulate et al. 2011)

Table 11 - Welch's approximate t-test Results on Mean Weight and SVL by Sex

Weight

Male SD	Female SD	DF	F ratio	t	prob>F
1.4	3.89	1	91.562	9.5688	<0.0001

SVL

Male SD	Female SD	DF	F ratio	t	prob>F
1.68	3.96	1	131.15	11.452	<0.0001

Table 11 - Welch's approximate t-test Results on Mean Weight and SVL by Sex - Due to the unequal variance between female and male data, a Welch's approximate t-test was used to compare mean SVL and weight between the sexes, finding significant sexual dimorphism in both cases.

Table 12 - Juvenile Morphology and Situation Found

Juveniles

Males	Sn-Vent (mm)	Leg Length (mm)	Head Width (mm)	Weight (g)	Site Code	Situation found
RP62	27.80	16.1	10.10	4.00	ML2	juvenile male, next to waters edge of rocky pool
RP63	34.50	16.2	10.10	6.60	ML2	juvenile male, rock in middle of stream
Means	31.15	16.2	10.10	5.30		

Females	Sn-Vent (mm)	Leg Length (mm)	Head Width (mm)	Weight (g)	Site Code	Situation found
RP78	34.20	16.2	10.70	5.00	NS	On top of riffle
RP80	32.40	15.8	10.80	5.00	NS	in large waterfall, damp portion of rock
Means	33.30	16.0	10.75	5.00		

Unable to Sex	Sn-Vent (mm)	Leg Length (mm)	Head Width (mm)	Weight (g)	Site Code	Situation found
RP56	31.70		9.10	3.50	ML1	Leaf litter 2m from water
RP66	25.80	11.4	9.80	2.60	DT	On rock at edge of stream, upstream of waterfall and alkaline tributary
RP68	22.00	14.1	7.60	0.50	DT	10cm from stream, upstream of waterfall and alkaline tributary
RP70	35.10	17.5	9.70	5.50	ML3	Under rock at edge of stream
RP71	32.40	16.4	10.80	5.50	ML3	On small tree branch overhanging stream
Means	29.40	14.9	9.40	3.52		
Total Juvenile Means	**30.66**	**15.7**	**9.86**	**4.24**		

Table 12 - Juvenile Morphology and Situation Found *- Details of the juvenile Snout-Vent length (mm), Leg length (mm), head width (mm), weight (g), site found and situation found, along with the means of morphological data. This data is separated by sex of juvenile, and then into those individuals which were unable to be sexed due to lack of sexual dimorphism in C.ranoides at a young age.*

No significant difference was observed in the SVL, leg length, head width or weight of the juvenile frogs measured between the sexes, and very little difference in morphometics was observed between the juvenile and adult male SVL or weight. This could be due to the increased probability of incorrect sex allocation of juveniles, at this stage of development.

Phylogenetic Analyses

Figure 4 - COI Phylogenetic Tree

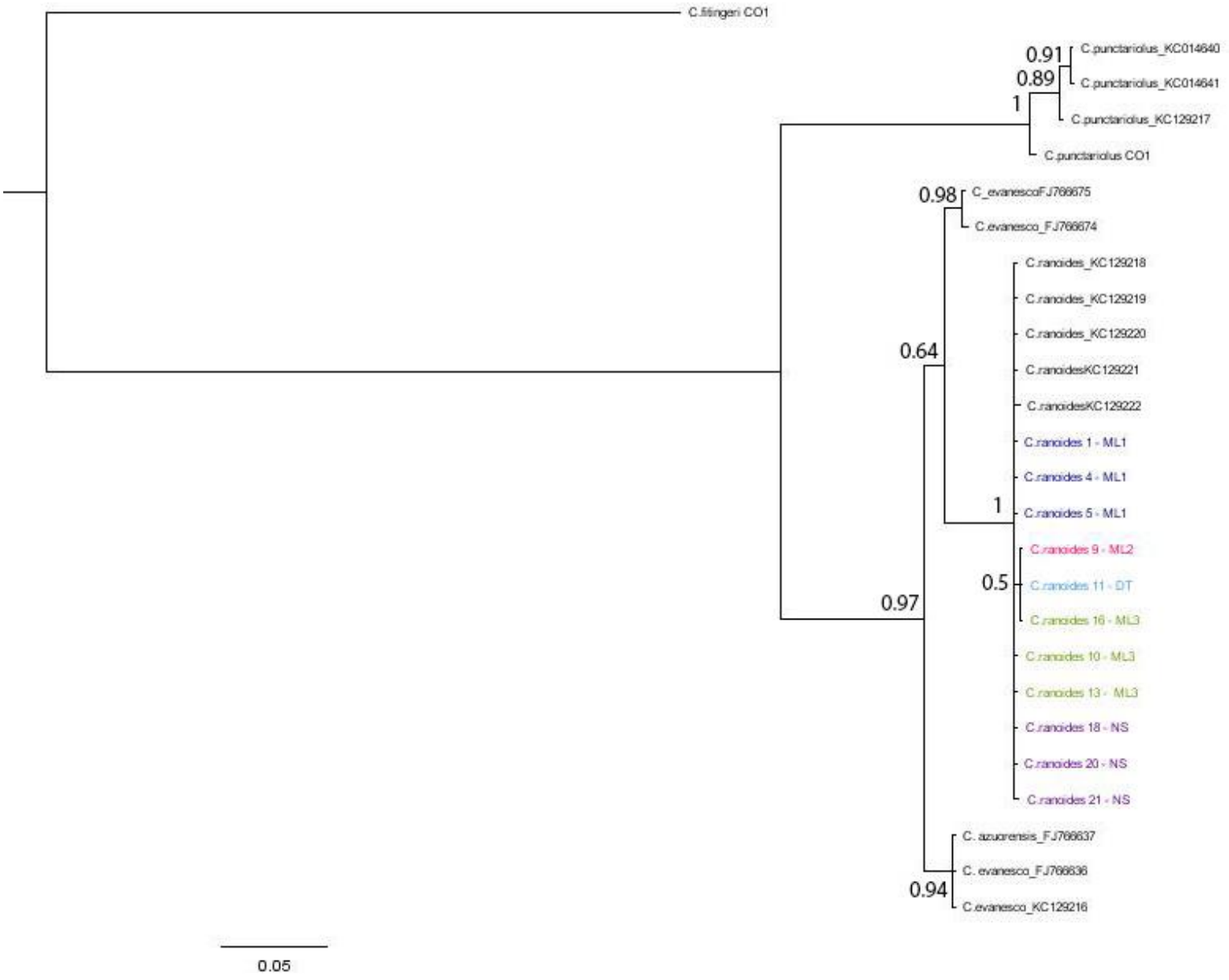

Figure 4 - COI Phylogenetic Tree - ***Phylogenetic relationships between 11 C.ranoides individuals collected across 5 sites, constructed using cytochrome oxidase subunit 1 gene sequence data***. *Posterior probabilities are included to depict branch support. Phylogeny also includes CO1 sequences from C.fitzingeri (outgroup), and other members of the Craugastor rugulosus species group; C.punctariolus, C.evanesco and C. azuorensis, along with five CO1 C.ranoides sequences from Genbank.*

The phylogenetic tree constructed using Bayesian inference methods, and CO1 sequence data, shows all eleven *C.ranoides* samples in this study collected in one supported clade (posterior probability – 1). The presence of a small unsupported clade (posterior probability 0.5) containing samples from three different sites is not thought to be significant in terms of speciation, as the maximum patristic distance between the sequences contained within it and any other *C.ranoides* sequence on the tree is 0.01 for each of the nodes (accession number KC12922, collected from the Rio Murcielago). All of the collected sequences in this study have a patristic distance of 0 between the smaller unsupported clade nodes and the wider clade of *C.ranoides*, further supporting the conclusion that all collected sequences from the five sites were the same species.

The separation of Genbank sequences of the species *C.evanesco* into two supported clades on the tree, based on CO1 sequence, suggests that misidentification of the individuals sampled may have occurred. Particularly as the two *C.evanesco* sequences grouped with *C.azueroensis* were collected from the same site as *C.azueroensis,* in Cocle, Panama, thus suggesting that these individuals were actually *C.azueroensis*. In the past, all of the *rugulosus* group species were considered to belong to one species; *C.rugulosus*, based on morphology alone, demonstrating the difficulty in identification of species encountered by those studying these species.

It is also possible that, due to the *C.evanesco* sequences in the later branching clade originating from a different site, genetic diversification may have occurred in the population in Veraguas, Panama, their site of collection. However, it is likely that many of the deeper branches in the *Craugastoridae* family remain unresolved, and would benefit from a study such as this, to solidify barcoding data. Such a study could attempt to separate *C.evanesco* from *C.azueroensis,* two species which overlap in distribution and are challenging to distinguish by morphology alone.

Table 13 - Species Delimitation Analysis based on CO1 sequences

Species	Closest Species	Mono-phyletic?	Intra-specific Distances	Interspecific Distances - Closest	Intraspecific distance/ Interspecific	P ID(Strict)	P ID(Liberal)	Av(MRCA-tips)	P (Randomly Distinct)	Clade Support	Rosenberg's P(AB)
C.punctariolus	*C.evanesco and C.azueroensis*	yes	0.015	0.219	0.07	0.82 (0.68,0.97)	0.97 (0.86,1.0)	0.0153	0.58	NA	0.0000053
C.evanesco	*C.evanesco and C.azueroensis*	yes	0.004	0.036	0.11	0.53 (0.38,0.68)	0.91 (0.76,1.0)	0.002	NA	0.00076	
C.ranoides	*C.evanesco*	yes	0.004	0.045	0.08	0.97 (0.91,1.0)	0.99 (0.96,1.0)	0.0018	NA	0.00076	
C.evanesco and C.azueroensis	*C.evanesco*	yes	0.002	0.036	0.07	0.75 (0.57,0.92)	0.97 (0.83,1.0)	0.0012	NA	0.00008	

Table11 – Species delimitation analysis based on CO1 sequences – *A distance based species delimitation analysis was conducted on the alignment of CO1 sequences used to create the CO1 phylogenetic tree, using the Geneious species delimitation plugin, which can be used to define species using the intraspecific/interspecific distance ratio. (Masters et al. 2011)*

In order to define the distinct clade of *C.ranoides* in this phylogenetic tree as one species, rather than simply false monophyly within what is truly a larger panmitic group of species; a species delimitation analysis was carried out on the CO1 sequence alignment. As the average pairwise tree distance between members of the *C.ranoides* species clade is low (0.004) it suggests a low level of genetic divergence in this group. This is also lower than the average pairwise tree distance between the members of the focal species and members of the next closest species (0.045). Thus, the intra/interspecific distance ratio (0.08) supports the definition of the *C.ranoides* samples collected from varying sites in Costa Rica as the same species, and the summation that they are the same species as the individuals sampled in Rio Murcielago in 2003.

Figure 5 - 16S Phylogenetic Tree

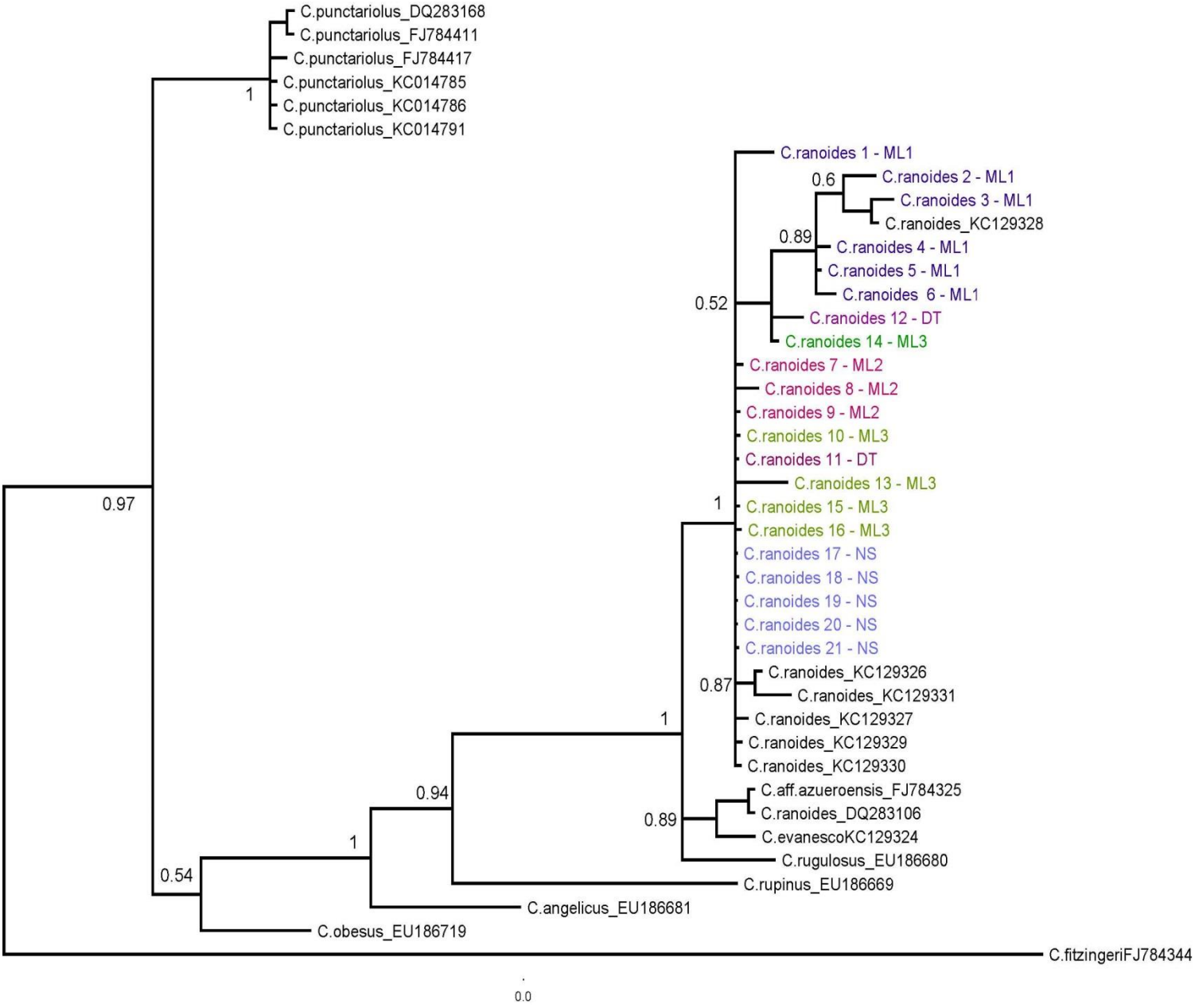

Figure 5 - 16S Phylogenetic Tree *Based on 16S sequences – Phylogenetic relationships between 21 C.ranoides individuals sampled, based on 16S rRNA sequence data. Tree also includes C.ranoides, C.rugulosus, C.rupinus, C.azueroensis, C.evanesco, C.punctariolus, C.angelicus, C.obesus and C.fitzingeri (outgroup) sequences from Genbank.*

The phylogenetic tree constructed from 16S sequence data seems to offer less concrete support for the hypothesis of a single species being sampled across the five sites included in this study. Indeed, it shows the first individual sampled; a juvenile from site 1 in the Murcielago sector, as genetically divergent in sequence from the other samples collected in

this study with posterior probability support of value 1. However, the largest patristic distance between this sample and any *Craugastor ranoides* sample is 0.03 (patristic distance between 1 and 3-adult male from ML1, and *C.ranoides* individual collected in Panama, accession number – DQ283106). These low genetic distances suggest that in fact this individual is of the species *C.ranoides*.

It is possible that this *C.ranoides* individual (1 – site ML1) was misidentified, potentially being of the species *C.azueroensis* (patristic distance 0.03 from *C.azueroensis* sequence in tree), however, due to the separation of this individual from *C.azueroensis* in the CO1 sequence data, and the low resolution of the surrounding groups, it is also likely that other *rugulosus* species phylogenies remain unresolved. This issue is not likely to be resolved with the study of historical data, but the collection of novel data from field studies, due to the lack of tissues belonging to the other *rugulosus* group species, and their preservation in formalin.

All other clades within the larger group of *C.ranoides* sequences have <0.95 posterior probability support, and therefore are considered to be insignificant in terms of species delimitation. Thus, the low genetic distance between the 16S sequences of the *C.ranoides* individuals sampled in this study supports a single species hypothesis. This low level of genetic divergence suggests that gene flow is maintained throughout these populations, and during the wet season, when moving across streams is enabled, inter breeding occurs.

Table 14 - Species Delimitation based on 16S sequences

Species	Closest Species	Monophyletic?	Intraspecific Distance	Interspecific Distance - Closest	Intraspecific/ Interspecific	P ID(Strict)	P ID(Liberal)	Av(MRCA-tips)	P (Randomly Distinct)	Clade Support	Rosenberg's P(AB)
C.punctariolus	*C.obesus*	yes	0.043	0.462	0.09	0.87 (0.75,1.00)	0.97 (0.87,1.0)	0.0231	NA	0.00000001	
C.ranoides	*C.ranoides*, *C.azueroensis* and *C.evanesco*	yes	0.115	0.266	0.43	0.86 (0.81,0.92)	0.96 (0.94,0.99)	0.0655	NA	0.00000210	
C.ranoides, *C.azueroensis* and *C.evanesco*	*C.rugulosus*	yes	0.089	0.265	0.34	0.57 (0.39,0.75)	0.82 (0.67,0.96)	0.0614	NA	0.00001400	
C.rugulosus	*C.ranoides*, *C.azueroensis* and *C.evanesco*	yes	0	0.265	0	0.00 (0.00,0.00)	0.96 (0.83,1.0)	0	NA	NA	0.00215
C.rupinus	*C.angelicus*	yes	0	0.823	0	0.00 (0.00,0.00)	0.96 (0.83,1.0)	0	NA	NA	0.00202
C.angelicus	*C.obesus*	yes	0	0.685	0	0.00 (0.00,0.00)	0.96 (0.83,1.0)	0	NA	NA	0.00189
C.obesus	*C.punctariolus*	yes	0	0.462	0	0.00 (0.00,0.00)	0.96 (0.83,1.0)	0	NA	NA	0.00178

Table 12 – Species delimitation analysis based on 16S sequences – *A distance based species delimitation analysis was conducted on the alignment of 16S sequences used to create the 16S phylogenetic tree, using the Geneious species delimitation plugin, which can be used to define species using the intraspecific/interspecific distance ratio.*

When the focal group selected was the clade containing *C.ranoides*, the average pairwise tree distance computed (0.115) was far greater than the average pairwise distance computed using C01 sequence data. However, this intraspecific distance was still smaller than the average pairwise distance between *C.ranoides* individuals and members of the closest clade (0.266). Thus, due to the low patristic distances observed between the *C.ranoides* sequence in a separate, supported clade on the tree (0.03), and those in the wider clade where separations are unsupported, the summation of *C.ranoides* as one species in the geographically separated sites still stands. However, some phylogeographic structure can be seen in the increased genetic distance of the ML1 individuals from the DT and NS site individuals, in comparison

to the ML2 and ML3 individuals. This could be due to the decreased distance which the more southern populations in the Murcielago sector have to move in order to interbreed with the populations on the other side of the Guanacaste Volcanic Chain, and therefore, a higher rate of gene flow between the closer populations.

More samples could investigate the higher level of genetic divergence observed in the 16S sequence data in comparison to the C01 sequence data, which may have been partially affected by the presence of the *C.ranoides* individual from Panama (DQ283106) in the closest clade, thus reducing the average pairwise genetic distance between the larger clade of *C.ranoides* and the clade containing *C.ranoides, C.azueroensis* and *C.evanesco.*

As observed in the CO1 sequence data, the *C.ranoides* Genbank sequences collected from the Rio Murcielago in 2003 (KC129328, 326, 327, 329, 330 and 331) fall into the same phylogenetic group as the sequences collected in this study, leading to the conclusion that correct identification of species occurred in both cases, and strengthening the use of 16S barcodes as an identification tool for *C.ranoides.*

Discussion

Using CO1 and 16S Sequences to Characterise Amphibian Diversity

The use of 16S and CO1 gene sequences in this study in order to characterise diversity, on the basis of previous studies which had successfully used these genes in the species delimitation of amphibians (Smith et al. 2008, Vences et al. 2005a), proved effective in allowing the clarification of the allopatric populations of *C.ranoides* as the same species. Despite the successful isolation of the CO1 gene sequence from the collected tissues proving challenging to accomplish in the laboratory, and requiring the specific alteration of the PCR recipe used, the gene proved able to distinguish between *rugulosus* group species genbank sequences, and grouped *C.ranoides* into one supported clade. However, significant trial and error of methodology was needed in order to achieve successful amplification of the CO1 gene region, with only 29% of gel bands on C01 PCRs showing correct amplification, in comparison to 96% for 16S.

16S *Craugastor ranoides* sequences seemed to display more genetic divergence. However, none of this divergence caused overlap between the intra-specific and interspecific variation in the species group, and thus, this gene was also determined to be sufficient to delimit species, fulfilling the requirements of a DNA barcoding marker for amphibians. The maintenance of this 'barcoding gap' (Hebert et al. 2003) is essential to the applicability of a gene for species identification using short sequences, as it suggests the balance needed between conservation and sufficient levels of divergence in the sequence is present.

The analysis of 16S and C01 sequence data from Genbank which were included in the phylogenetic trees in this study revealed the need for further resolution of the *rugulosus* species group taxonomy. Particularly due to the grouping of sequences from Veraguas, Panama, which were recorded as *C.evanesco* and *C.azueroensis,* into one species group.

Further studies such as this could attempt to separate *C.evanesco* from *C.azueroensis*, along with other sympatric, and closely related, though not identical, species which are difficult to distinguish using morphology alone. The addition of nuclear genetic sequence data to these analyses such as this could help to add resolution to the deeper relationships in the tree, and thus, clarify taxonomy in the wider rugulosus *species* series.

This difficulty in the identification of amphibian species by morphology alone was demonstrated by the non-confirmation of some of the data collected on this study to the previous percentage ranges for morphometric data described in 2000. (Campbell & Savage, 2000) Female head width as a percentage of snout vent length did not fall into the previously described range in any of the sampled individuals, which could have led to a hypothesis of species divergence in the absence of genetic data. This also illustrates the importance of the integration of data sources such as calls, and ecology into species determination in these relictual populations.

Distribution of Relictual Populations

The confirmation of the persistence of *C.ranoides* in the Murcielago sector of the Santa Elena peninsula and the Quebrada la Danta, which was confirmed in this study with the sighting of breeding populations, gives support to the hypothesis that the conditions in these regions protect this species from the damaging effects of chytrid disease. In a model constructed to detail the distribution of *Bd* in Costa Rica, the climatic profile of the regions where *C.ranoides* persists were found not to overlap with any infected site. (Puschendorf et al. 2009) This suggests that the high temperatures and six month dry season of the Santa Elena Peninsula enable *C.ranoides* persistence, in comparison to its decline in the nearby, but colder and more humid Guanacaste Volcanic chain.

Watercourses in the dry forest of the Santa Elena Peninsula which remain unexplored show the high temperatures and six month dry season (Puschendorf et al. 2005) necessary to provide a refuge from chytrid disease to amphibian species. (Zumbado-Ulate et al. 2007) Such areas in countries such as Costa Rica have high probabilities of successful rediscovery of relict populations. Maps which predict the most likely areas of re-discovery in Costa Rica show that most of these areas have not been sampled since 2000 (González-Maya et al. 2013). One such site, the Rio Nisperal, is housed in the Naranjo valley, where this study recorded the newly found presence of *C.ranoides.* Due to the high level of potential biodiversity in areas such as Rio Nisperal, their identification and protection is crucial to the further conservation of biodiversity, and ensuring that potential relocation proposals to conserve other species succeed. (Woodhams et al. 2011)

16S and CO1 sequence data in this study confirmed that the *C.ranoides* individuals in the Rio Nisperal were not genetically divergent from the populations in the other sites. Thus, this population is likely to represent the remnants of the previous larger distribution of *C.ranoides*, ranging through higher altitude montane areas, which has been separated from the other populations by the rising prevalence of *Bd* in the Guanacaste Volcanic Range. However, the increasing conservation of genetic sequence in the more northern sites indicates that a gradient of increasing levels of gene flow is occurring between the populations of *C.ranoides* in the Santa Elena Peninsula with decreasing distance between them. Thus, some interbreeding is likely to be occurring in the wet season between these seemingly allopatric populations. (Zumbado-Ulate et al. 2011)

In contrast to the confirmation of *C.ranoides* persistence in the Murcielago sector and Quebrada la Danta, no *C.ranoides* individuals were found in the Rio la Calera, where *C.ranoides* had previously been recorded. (Zumbado-Ulate et al. 2007) This was thought to be due to the low river level observed, and its allowance of larger mammals such as tapirs to

inhabit this watercourse, evidence of which was seen in faeces. This eutrophication of the natural habitat of *C.ranoides* had led to large amounts of algal growth in the river, showing the dangerous effects of climate change in such pristine environments. Species of the family *Ranidae* which had not previously been seen by Robert Puschendorf were also sighted in this area, a sign of changing environmental conditions. In January 2015, the stream was found to be flowing once again, but *C.ranoides* individuals were still not sighted, and Ranids still persisted. (Puschendorf, *pers.obs.*) Such observations serve as a reminder that chytrid disease is only one of the environmental factors threatening amphibian populations in pristine habitats. Climate change has the dangerous ability to only temporarily lower water levels, but permanently exclude an amphibian species from the site, due to their need for constantly flowing streams.

Translocation of *C.ranoides*

In order to capitalise on the protective environment of the Santa Elena peninsula, and the persisting populations of *C.ranoides* which it harbours, translocations from the five relictual populations in this study to former *C.ranoides* range areas have been proposed. These areas include nearby Volcan Cacao (Figure 1). Translocation, the release of animals into former areas within their range, has been evaluated continually as an appropriate conservation strategy for amphibians for the past 20 years. In a review of the results of translocation projects of amphibians and reptiles initiated between 1991 and 2006, the success rate of the projects was found to have doubled since an earlier review in 1991, an encouraging statistic for conservationists who look to translocations as a way of protecting native populations under threat from disease and habitat loss. (Dodd and Seigel 1991, Germano and Bishop 2009, Zeisset and Beebee 2013) Indeed, in 2007 the Amphibian Conservation Summit listed translocations as one of the three long term conservation programmes requiring development and implementation in the Amphibian Conservation Action Plan. (Gascon et al. 2007)

Along with the long term commitment of National Parks and conservationists which is required for translocation projects to succeed, closely related donor populations have been shown to increase their success, due to the reduced risk of the introduction of maladapted genotypes into the new range. (Zeisset and Beebee 2013) Thus, the support this study provides to the hypothesis that the five geographically separated populations of *C.ranoides* in the Santa Elena peninsula are in fact genetically indistinct, due to continued gene flow, allows these translocation plans to move forward with greater assurance of success. Translocations from the sites across the Santa Elena peninsula will have a smaller chance of generating outbreeding depression due to the small genetic distance between the allopatric populations. This increased chance of success in well directed active conservation strategies, along with the continued protection of climatic refugia for amphibian species, will help to ensure the protection of relictual populations of amphibians, such as *C.ranoides*.

Conclusion

Due to the absence or negligible level of genetic divergence between the allopatric populations of *C.ranoides* sampled, the results of this study lead to the conclusion that they do indeed represent the same species. Species delimitation analyses on 16S and CO1 sequence data supported this conclusion based on the low level of intraspecific divergence in comparison to interspecific divergence found in these sequences when compared with other *rugulosus* group clades. However, some of the morphological data collected did not conform to previous identifying characters of the species (HW % of SVL) and thus, the identification of this species group by morphology alone is not recommended.

More species specific studies on the natural history and ecology of the *rugulosus* group are urgently needed. This should be accompanied by the collection of genetic data to submit to barcoding databases, in order to clarify some of the putative misidentification of species which was seen in the sequences taken from Genbank to use as outgroups in this study.

On the basis of this research, proposals to translocate some *C.ranoides* individuals from their relictual breeding grounds in the Murcielago sector, Quebrada la Danta and Rio Nisperal to their previous habitats in areas such as Volcan Cacao, gain further support. This is due to the clarification of single species status on the separated populations, and thus, the greater assurance of translocation of the correct species. In addition, the presence of some phylogeographic structure in the 16S sequence data suggests that movement across the higher altitudes will predispose the translocated individuals to adaptation.

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Huge thanks are owed to Dr. Robert Puschendorf for all his advice and assistance with this project, in the field, in the lab and when writing the report, along with Prof. Alex Rogers and Timothy Walker for their helpful advice in the writing up stages. I am grateful for the help of the staff at the Area de Conservation Guanacaste, especially Maria Marta Chavarria, for driving us into the Santa Elena Peninsula restricted areas. This project would not have been possible without the IUCN and the Area de Conservation Guanacaste allowing us to remove the tissue samples from Costa Rica, for which I am also grateful.

Management Report

Having previously worked on a short amphibian field work project in Costa Rica in the summer of 2013, I was keen to conduct an amphibian study as part of my FHS project. Dr. Robert Puschendorf of Plymouth University agreed to supervise this project in MT13, and we discussed the design of the project during the Christmas holidays and HT14. Due to the difficulties encountered with real time PCR analysis, the decision was made not to focus on *Batrachochytrium dendrobatidis* directly in the study, but to test the genetic diversity of relictual populations of frogs which had persisted despite being surrounded by *Bd* prevalent areas.

The majority of TT14 was spent reading relevant literature, which was recommended by Dr. Puschendorf, however, the project design could not be finalised until we reached the Santa Elena peninsula in June 2014, due to environmental conditions determining the presence or absence of species in this area. The collection of tissue samples and morphological data was conducted across seven sites in the Santa Elena peninsula in June and July 2014, two of which disappointingly revealed no populations of *C.ranoides*. Swabs of the individuals samples were also taken for the presence or absence of *Bd*, however, time constraints did not allow the analysis of these samples, and thus they remain in the freezer at Plymouth University, and could be used in later studies.

The laboratory analysis, including the extraction of DNA and CO1 and 16S PCR, was conducted between the 26th of August and the 7th of September 2014, at Plymouth University. Although the 16S PCR worked well, I had difficulty achieving correct amplification of the CO1 gene, and had to repeat the protocols with revised recipes after research into the methods used in other amphibian studies. After the PCR products were sent for sequencing at

MACROGEN, only half of the CO1 gene samples had amplified to a useable resolution, however, as one sample had correctly amplified per site, the analysis could still be completed.

I completed a detailed report of my methods in the lab and field at the beginning of MT14, and spent MT14 completing a literature review, along with beginning the analysis of my chromatographic data, which I received during MT14. During the Christmas holidays, I conducted the analysis of the genetic data and construction of phylogenetic trees, and later added species delimitation analyses, on recommendation from Prof Alex Rogers. I then analysed my results in order to complete my project report at the beginning of HT15.

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Supplementary Information

Table 15- Details of Sampling by Date

Date	Site	No. of C.ranoides sighted	Total samplers present	Time began sampling	Time ended	No. not stopping to measure	Any deductions for all measuring (mins)	Time of first frog	time to first frog x total number of samplers (mins)	time after first frog (mins)	Total sampling time = (time to first frog x total number of samplers) + (time remaining x samplers - 2) - any deductions
27/06/14	ML1	6	4	18:35	20:56	2	20	18:45	40	131	282
27/06/14	ML2	3	4	21:32	22:29	2	0	21:56	96	33	162
27/06/14	ML3	1	4	23:02	23:45	2	0	23:40	152	5	162
28/06/14	DT	4	4	18:51	20:45	2	0	18:55	16	110	236
28/06/14	CL	-	4	21:30	22:20	2	0	none sampled	200	-	200
29/06/14	ML3	6	4	19:00	20:18	2	0	19:15	60	63	186
30/06/14	CC	-	4	20:21	21:18	2	0	none sampled	228	-	228
01/07/14	NS	18	3	18:48	20:18	3	0	19:42	162	36	270

Table 16 - Original CO1 PCR Recipe

COI	per 10uL	per 20uL	25x20uL	Plate (105)	conc.
dgHCO2198	0.30	0.60	15.00	31.50	10uM stock
dgLCO1490	0.30	0.60	15.00	31.50	10uM stock
10X buffer	1.00	2.00	50.00	105.00	1X final
dNTPs	0.50	1.00	25.00	52.50	2mM/nuc stock
Mg2+ (addl)	0.20	0.40	10.00	21.00	25mM/uL stock
BSA	0.25	0.50	12.50	26.25	10mg/mL stock
Taq	0.10	0.20	5.00	10.50	5 units/uL
ddH20	6.35	12.70	317.50	666.75	
gDNA	1.00	2.00	50.00		
Total uL:	10.00	20.00	500.00	945.00	*uL*

Table 17 – 16S PCR Recipe

16S	per 10uL	per 20uL	20uLx25	Plate (105)	conc
16SB-H (16Sbr-H)	0.30	0.60	15.00	31.50	10uM stock
16SA-L (16Sar-L)	0.30	0.60	15.00	31.50	10uM stock
10X buffer	1.00	2.00	50.00	105.00	1X final
dNTPs	0.50	1.00	25.00	52.50	2mM/nuc stock
Mg2+ (addl)	0.20	0.40	10.00	21.00	25mM/uL stock
Taq	0.10	0.20	5.00	10.50	5 units/uL
ddH20	6.60	13.20	330.00	693.00	
gDNA	1.00	2.00	50.00		
Total uL:	10.00	20.00	500.00	945.00	uL

Table 18 - C01 Recipe A (with BSA)

C01 Recipe A (with BSA)	per 10uL	13x10uL
dgHCO2198	0.30	3.90
dgLCO1490	0.30	3.90
10X buffer	1.00	13.00
dNTPs	0.50	6.50
Mg2+ (addl)	0.20	2.60
BSA	0.25	3.25
Taq	0.10	1.30
ddH20	6.35	82.55
gDNA	1.00	13.00
Total uL	10.00	130.00

Table 19 - C01 Recipe B (without BSA)

C01 Recipe B (without BSA)	per 10uL	25x10uL
dgHCO2198	0.30	7.50
dgLCO1490	0.30	7.50
10X buffer	1.00	25.00
dNTPs	0.50	12.50
Mg2+ (addl)	0.20	5.00
Taq	0.10	2.50
ddH20	6.60	165.00
gDNA	1.00	25.00
Total uL	10.00	250.00

Table 20 - CO1 Recipe C (with 4.8uL BSA per 10uL)

CO1 Recipe C (with 4.8uL BSA per 10uL)	per 10uL	12x10uL
dgHCO2198	0.30	3.60
dgLCO1490	0.30	3.60
10X buffer	1.00	12.00
dNTPs	0.50	6.00
Mg2+ (addl)	0.20	2.40
BSA	0.48	5.76
Taq	0.10	1.20
ddH20	6.12	73.44
gDNA	1.00	12.00
Total uL	10.00	120.00

Table 21 – PCR Reaction: COI

PCR Reaction: COI	Time	Temp
1) Initial melt	3 min	94°C
2) Denaturation	30 sec	94°C
3) Annealing	30 sec	49°C
4) Extension	40 sec + 1 cycle	72°C
5) Goto 2	33 times	x
6) final Extn.	10 mins	72°C
7) Refridgerate	infinite	10°C

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