Herpetology Notes, volume 11: 97-100 (2018) (published online on 27 January 2018)

A molecular protocol to distinguish syntopic Galápagos land iguanas (*Conolophus marthae* and *C. subcristatus*) from faecal samples

Livia Di Giambattista[1] and Gabriele Gentile[1,*]

Abstract. *Conolophus marthae* is a critically endangered, rare, and hard-to-sample land iguana from the Galápagos Islands of Ecuador that lives syntopically with *C. subcristatus*. Unfortunately, information about this iconic species is still lacking, and critical research needs include monitoring of populations and habitat trends, as well as dietary analysis. We developed and applied a molecular protocol to rapidly assign *Conolophus* faeces to the correct species of Galápagos land iguana. The protocol includes PCR amplification of a short region of the mtDNA control region, followed by a selective digestion with the restriction enzyme AvaII. The protocol is relatively inexpensive and easy to use and proved 100% efficient when used with fresh faeces, while its efficiency decreased to 17% for faeces exposed to the environment for several days or weeks.

Keywords: Faecal DNA, scat, molecular tool, AvaII, species determination, non-invasive method, RFLP, PCR, Iguanidae, diet

Introduction

The only existing population of the Galápagos pink iguana (*Conolophus marthae*; Fig. 1A) lives on the northwestern slope of Wolf Volcano, in the northern part of Isabela Island (Fig. 2), where it lives in syntopy (*sensu* Rivas, 1964) with a population of the congeneric *C. subcristatus* (Fig. 1B). Whereas both species are endemic to the Galápagos Archipelago, *C. subcristatus* is widely distributed across the islands, whereas *C. marthae* has never been observed outside an area larger than 25 km²; the exact range of the species still remains to be investigated. The habitat of the species includes areas with tropical dry shrubs at the top of the volcano and tropical dry forest along its slopes. *Conolophus marthae* is listed as critically endangered in the IUCN Red List (Gentile, 2012), and much basic information about this species is still lacking, mainly due to the difficulties in carrying out prolonged fieldwork and data collection.

When direct observation is difficult and trapping is not possible due to conservation concerns, as in the case of *C. marthae*, the use of indirect methods becomes critically important. In fact, the assignment of faeces to the correct species would allow us to gain data about the presence of *C. marthae* in peripheral areas, where direct sightings may be less frequent. Additionally, once faeces have been assigned to the correct species, they can be inspected by means of traditional microscopic analysis for the presence of plant material, seeds, and animal remains (Soininen et al., 2009). The identification of seeds and arthropods would contribute valuable information regarding the diet of the species (Traveset et al., 2016).

Non-invasive wildlife research using DNA extracted from faeces has become increasingly popular (Schwartz and Monfort, 2008). While this approach would certainly be very useful for *C. marthae*, given that faeces of the two congeneric species are morphologically undistinguishable, some technical problems exist (Smith et al., 2000). The main difficulty with DNA extraction from faeces lies in the low quantity and quality of extractable DNA (Buchan et al., 2005). The influence of low DNA quantity and quality on the efficiency of genetic data collection and analysis increases when faecal samples are exposed to the environment for an extended time prior to collection (Buchan et al., 2005;

[1] Department of Biology, University of Rome Tor Vergata, I-00133 Rome, Italy
* Corresponding author. E-mail: gabriele.gentile@uniroma2.it

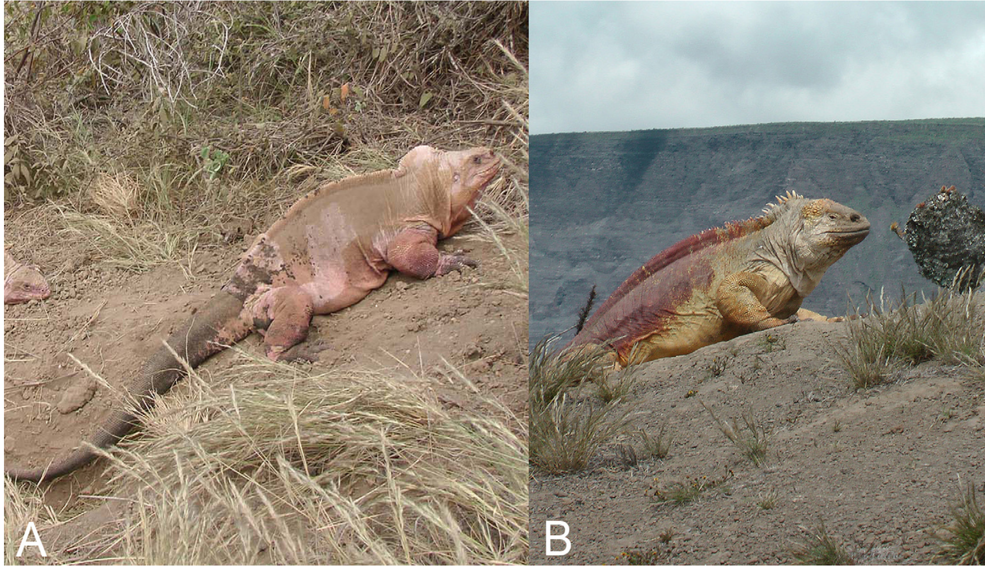

Figure 1. Males of (A) *Conolophus marthae* and (B) *C. subcristatus* from Wolf Volcano, Isabela Island, Galápagos, Ecuador. Photos by G. Gentile.

Waits and Paetkau, 2005; Ball et al., 2007; Murphy et al., 2007). When the goal is to obtain evidence for the presence of a species, the use of short fragments of mitochondrial DNA offers a suitable efficiency in the analysis due to the higher copy number of mtDNA in cells (Hebert et al., 2003; Waugh, 2007).

Figure 2. Map of the Galápagos Islands, Ecuador. The black triangle indicates the location of Wolf Volcano on Isabela Island. Islands with land iguanas, or where land iguanas occurred in the past, are shaded in grey. Crosses indicate extinctions in historic times.

In order to assign samples to the right species unambiguously, we developed a molecular protocol to distinguish faecal samples released on the ground by *C. marthae* or *C. subcristatus*. We used freshly released as well as dry faecal samples collected in the field to check for the general applicability of the protocol to faeces for which the time elapsed from release to collection is unknown but may span from several days to weeks before deteriorating.

Materials and Methods

Entire faeces were collected along an altitudinal range between 1500 and 1700 m on Wolf Volcano, in the northern part of Isabela Island, Galápagos. Samples were opportunistically collected on the ground during three sampling seasons (July 2010, June 2012, June 2014) in tropical dry shrub-dominated habitat.

Thirty fresh samples were taken from identified individuals. These samples (15 from *C. marthae*, 15 from *C. subcristatus*) were used to test the efficiency of the molecular protocol on freshly released faeces. One hundred and thirty-four unidentified samples were also collected. The elapsed time from release to collection of these faeces was unknown and could vary from days to weeks. In fact, in the field faeces fully deteriorate after a few weeks. Each faecal sample was preserved

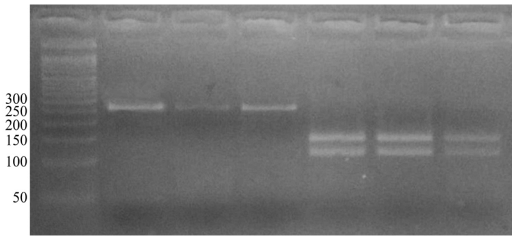

Figure 3. Amplicons resulting from digestion by the enzyme AvaII. Single and double bands indicate *C. subcristatus* (Lanes 2–4) and *C. marthae* (Lanes 5–7), respectively. The number of base pairs can be inferred from the DNA ladder run in Lane 1 on the left side of the gel.

in isolation from other samples to avoid inter-sample contamination. Independent of their age, faeces were immediately frozen and stored at –10°C while in the field and at –20°C in the laboratory, until they were processed.

Genomic DNA was extracted from each sample by cutting out about 1 g from the external surface of each faeces. DNA extraction was performed using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany; qiagen.com), according to the protocol in QIAGEN (2010).

We amplified a short fragment of the mtDNA control region. To design appropriate primers, we used aligned sequences of this region. The dataset comprised several sequences of both *C. marthae* and *C. subcristatus*, obtained from previous phylogenetic and phylogeographic studies (Gentile et al., 2009; Ciambotta et al., 2013). Primers were designed to PCR-amplify in both species a fragment of short length (261 bp). Primer sequences were: Forward, ACGCGAGAAATCAGCATCC; Reverse, TATGYGGGACTGAAGCCAA. The *C. marthae* amplicon included a diagnostic restriction site for the enzyme AvaII at position 112 of the PCR fragment.

PCRs were performed using Master Mix 2X (Promega, Madison, Wisconsin, USA; promega.com) according to the manufacturer's protocol. The amplification protocol was: 94°C (5 min), initial denaturation; 40 cycles with 94°C (30 s) denaturation, 57°C (45 s) annealing, 72°C (45 s) elongation; 72°C (7 min) final elongation. Each sample was amplified three times to avoid possible random non-amplification. The PCR fragments were then used as template for the restriction enzyme AvaII (Promega, Madison, Wisconsin, USA; promega.com). AvaII is a 5' overhang cutter enzyme that recognises the 5 bp long sequence GGWCC and cuts between the two guanines. Because only the amplicon from *C. marthae* includes the sequence GGACC (the corresponding sequence in *C. subcristatus* is GGGCC) only amplicons belonging to *C. marthae* would be cut into two fragments (112 and 149 bp) after digestion by AvaII. As with other endonucleases, AvaII is sensitive to overlapping Dcm methylation that can block endonuclease activity (Riegel and Nassal, 1993). However, this is irrelevant for our purposes as amplicons obtained by PCR do not retain a DNA methylation profile.

Restriction reactions were performed according to the protocol of the manufacturer, incubating at 37°C in Buffer C, to promote higher efficiency. RFLP patterns were scored using a UV transilluminator, after 40 min of electrophoresis in a 4% agarose gel. The protocol was initially tested on the 30 freshly released faecal samples collected from identified individuals.

Results

The protocol allowed us to unambiguously distinguish syntopic Galápagos land iguana species using stool samples. The efficiency of extraction, amplification, and digestion for fresh control samples was 100%. Restriction fragments were discriminated well by the agarose gel (Fig. 3). RFLP analysis resulted in the correct species assignment for all samples. However, of the 134 unidentified samples collected on the ground, only 23 were successfully amplified and digested (efficiency of 17.2%). Of these, 21 were assigned to *C. subcristatus* and two to *C. marthae*.

Discussion

Hart et al. (2015) recently compared different extraction methods from stool samples when using manufacturers' instructions. They showed that different protocols might perform differently and selectively when applied to samples from specific host species. These authors also observed that spectrophotometric assessment of DNA elution prior to PCR would not predict successful amplification. In our case, the efficiency was high when faeces had been freshly released but decreased significantly when the time elapsed since defecation increased to several days or weeks. A combination of factors, such as temperature, weather conditions, or high UVB radiation – measured as high as 500–600 $\mu W/cm^2$ – may affect the possibility to recover amplifiable DNA. Even though the addition of bovine serum albumin in the PCR mix could improve the rate of amplification,

in our case the quality and quantity of DNA contained in each sample is strongly affected by the time of exposure to the environment. Despite this, as the protocol is relatively inexpensive and easy to apply, it may represent a valuable tool to aid programs aimed to obtain information about the geographic occurrence of the species, to complement direct capture or sighting.

Additionally, the possibility to distinguish *C. marthae* and *C. subcristatus* by their faeces, even at the low levels of success obtained when the time since defecation is greater than a few days, makes our method a necessary complement to traditional faeces examination when attempting a dietary assessment of *C. marthae*. Our protocol allows for a completely non-invasive approach, whereas alternative techniques, such as stomach flushing, can negatively affect the survival of lizards (Luiselli et al., 2011). Diet assessment is important to evaluate competition for trophic resources that may exist between the two species (Gentile et al., 2016) and for the purposes of captive breeding or headstart programs.

Acknowledgments. This work is part of a long-term collaborative programme between the Galápagos National Park and the University of Rome Tor Vergata, aimed at the conservation of Galápagos land iguanas. We thank P. Jaramillo, M. Onorati, W. Tapia, and Galápagos National Park rangers for helping collect and process faecal samples. Funding was provided by the Mohamed bin Zayed Species Conservation Fund (project number 12254183; http://www.speciesconservation.org/case-studies-projects/galapagos-pink-land-iguana/4183).
We are grateful to Mark Auliya and Hinrich Kaiser for their constructive criticism on an earlier version of this work.

References

Ball, M.C., Pither, R., Manseau, M., Clark, J., Petersen, S.D., Kingston, S., Morrill, N., Wilson, P. (2007): Characterization of target nuclear DNA from faeces reduces technical issues associated with the assumption of low-quality and quantity template. Conservation Genetics **8**: 577–586.

Buchan, J.C., Archie, E.A., van Horn, R.C., Moss, C.J., Alberts, S.C. (2005): Locus effect and sources of error in noninvasive genotyping. Molecular Ecology Notes **5**: 680–683.

Gentile, G., Fabiani, A., Marquez, C., Snell, H.L., Snell, H.M., Tapia, W., Sbordoni, V. (2009): an overlooked pink species of land iguanas in the Galápagos. Proceedings of the National Academy of Sciences USA **106**: 507–511.

Gentile, G. (2012): *Conolophus marthae*. The IUCN Red List of Threatened Species 2012. Available at http://www.iucnredlist.org/details/174472/0. Accessed on 10 February 2017.

Gentile, G., Marquez, C., Tapia, W., Izurieta, A. (2016): Conservation of a new flagship species: the Galápagos Pink Land Iguana (*Conolophus marthae* Gentile and Snell, 2009). In: Problematic Wildlife, a Cross-Disciplinary Approach, p. 315–336. Angelici, F.M., Ed., New York, USA, Springer International Publishing.

Hart, M.L., Meyer, A., Johnson, P.J., Ericsson, A.C. (2015): Comparative evaluation of DNA extraction methods from feces of multiple host species for downstream next-generation sequencing. PLoS ONE **10 (11)**: e0143334.

Hebert, P.D.N., Cywinska, A., Ball, S.L., de Waard, J.R. (2003): Biological identification through DNA barcodes. Proceedings of the Royal Society of London B **270**: 313–321.

Luiselli, L., Akani, G.C., Ebere, N., Pérez-Mellado, V. (2011): Stomach flushing affects survival/emigration in wild lizards: a study case with rainbow lizards (*Agama agama*) in Nigeria. Amphibia-Reptilia **32**: 253–260.

Murphy, M.A., Kendall, K.C., Robinson, A., Waits, L.P. (2007): The impact of time and field conditions on brown bear (*Ursus arctos*) faecal DNA amplification. Conservation Genetics **8**: 1219–1224.

QIAGEN (2010): QIAamp® DNA Stool Handbook. Second Edition. Hilden, Germany, QIAGEN. 43 pp.

Rivas, R.L. (1964): A reinterpretation of the concepts “sympatric” and “allopatric” with proposal of additional terms “syntopic” and “allotopic”. Systematic Zoology **13**: 42–43.

Schwartz, M.K., Monfort, S.L. (2008): Genetic and endocrine tools for carnivore surveys. In: Noninvasive Survey Methods for Carnivores, p. 238–262. Long, R.A., Mackay, P., Zielinski, W.J., Ray, J.C., Eds., Washington, D.C., USA, Island Press.

Smith, K.L., Alberts, S.C., Bayes, M.K., Bruford, M.W., Altmann, J., Ober, C. (2000): Cross-species amplification, non-invasive genotyping, and non-Mendelian inheritance of human STRPs in savannah baboons. American Journal of Primatology **51**: 219–227.

Soininen, E.M., Valentini, A., Coissac, E., Miquel, C., Gielly, L., Brochmann, C., Brysting, A., Sønstebø, J.H., Ims, R.A., Yoccos, N.G., Taberlet, P. (2009): Analyzing diet of small herbivores: the efficiency of DNA barcoding coupled with high-throughput pyrosequencing for deciphering the composition of complex plant mixtures. Frontiers in Zoology **6**: 16.

Traveset, A., Nogales, M., Vargas, P., Rumeu, B, Olesen, J.M., Jaramillo, P., Heleno, R. (2016): Galápagos land iguana (*Conolophus subcristatus*) as a seed disperser. Integrative Zoology **11**: 207–213.

Waits, L.P., Paetkau, D. (2005): Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. Journal of Wildlife Management **69**: 1419–1433.

Waugh, J. (2007): DNA barcoding in animal species: progress, potential and pitfalls. Bioessays **29**: 188–197.

Accepted by Hinrich Kaiser