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Development of a molecular identification protocol for roadkill birds in San Cristóbal-Galapagos.

Renato Sebastián Morales Acuña

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Renato Sebastián Morales Acuña

Nombre del profesor, Título académico **Juan José Guadalupe, MSc.**

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Nombres y apellidos: Renato Sebastián Morales Acuña

Código: 00212533

Cédula de identidad: 1723068159

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RESUMEN

Se conoce por estudios previos una alta tasa de mortalidad en Galápagos, sin embargo, no se tiene información de ciertas islas; además los estudios se encuentran limitados debido a que existe un alto porcentaje de muestras irreconocibles que no son utilizadas para identificación morfológica. Según estudios previos, existe alta mortalidad en la especie Canario María y en diversas especies de pinzones. El presente estudio tiene como objetivo diseñar un protocolo de identificación molecular para aves atropelladas en la isla San Cristóbal en Galápagos con el fin de utilizar está clase de muestras. Para esto se realizó un protocolo de DNA barcoding en base a la identificación de las regiones Citocromo oxidasa I (COI) y Citocromo B (CytB). Los resultados obtenidos muestran que el protocolo es eficiente para separar entre familias de aves e identificar especies, y en el caso de los pinzones, se logró llegar a identificar hasta género. Estos resultados permiten concluir que el protocolo es útil para la identificación de muestras de aves atropelladas que son irreconocibles. Además, el protocolo es aplicable para las demás islas del archipiélago de Galápagos.

Palabras clave: Pinzones, DNA barcoding, conservación, protocolo, atropellamientos de vida silvestre

ABSTRACT

Previous studies have documented a high mortality rate in Galápagos; however, there is limited information available for certain islands. Additionally, studies are constrained by a high percentage of unidentifiable samples that are not utilized for morphological identification. Previous research has highlighted high mortality rates among the yellow warbler species and various finch species. This study aims to design a molecular identification protocol for road-killed birds on San Cristóbal Island in Galápagos, specifically targeting these unidentifiable samples. To achieve this, a DNA barcoding protocol based on the identification of the Cytochrome oxidase I (COI) and Cytochrome B (CytB) regions was implemented. The results demonstrate the protocol's efficacy in distinguishing between bird families and identifying species, with the ability to even identify genera in the case of finches. These findings lead to the conclusion that the protocol is valuable for identifying unidentifiable samples of road-killed birds. Furthermore, the protocol is applicable to other islands within the Galápagos archipelago.

Key words: Finches, DNA barcoding, conservation, protocol, wildlife roadkill.

TABLA DE CONTENIDO

ÍNDICE DE TABLAS

INTRODUCTION

Roads and transportation networks plays an important role in communities by enabling cost reduction in transportation, boosting tourism, facilitating transportation, enhancing mobility between different regions, and improving accessibility to territories (Alqershy et al., 2023). However, in various ecosystems, roads can cause habitat disturbance due to noise or artificial light or ecosystem fragmentation (Seiler, 2001) resulting in barrier effects that contribute to wildlife mortality on roads (Morelli et al., 2014).

Roadkill represents the second leading anthropogenic cause of vertebrate mortality (Schwartz et al., 2020). Birds, in particular, are significantly impacted by this phenomenon; in the United States, it is estimated that between 89 to 340 million birds perish annually due to collisions with vehicles (Lala et al., 2021). Similarly, Ecuador exhibits a comparable trend, with birds being one of the most common victims to roadkill incidents (Medrano-Vizcaíno et al., 2023). A study conducted on Santa Cruz Island within the Galapagos archipelago revealed high avian mortality rates along two road segments (García-Carrasco et al., 2020), underscoring the severity of this issue.

In Galapagos, one of the factors contributing to the increase in wildlife roadkill incidents is the growth in population and tourism. Over the last 30 years, the population has steadily increased; in 1990, approximately 10,000 people inhabited the Galapagos Islands (Gardener & Grenier, 2011), by 2001, the population had risen to 18,640 (Ragazzi et al., 2016), and by 2016, it had reached 25,224 (García-Carrasco et al., 2020). This demographic trend typically accompanies the expansion of roads and transportation networks (Gonzáles & Turner, 2018). Furthermore, the rise in population leads to an increase in automobiles, which escalated from 28 to 1,074 on Santa Cruz Island between 1980 and 2006 (García-Carrasco et al., 2020).

Additionally, tourism has surged in recent years; from 2021 to 2022, there was an astounding 71% increase in tourist arrivals to San Cristóbal Island (Ministerio de Turismo, 2022),

resulting in heightened vehicular traffic and consequently raising the likelihood of wildlife roadkill incidents.

In the study conducted on Santa Cruz Island, the most impacted avian species was *Setophaga petechia* (yellow warbler) (García-Carrasco et al., 2020). *Setophaga petechia* is a small migratory passerine bird that ranges from northern Alaska to central Peru; however, the subspecies found in the Galapagos Islands is non-migratory (McAllister & Hnida, 2022). It is characterized by its yellow plumage, although at a young age, it often exhibits a grayish coloration that can be mistaken for the plumage of other finches, especially given their similar size (The Cornell Lab of Ornithology, 2019).

Another group of species highly affected by roadkill in Galapagos were the Darwin's finches (García-Carrasco et al., 2020). Finches constitute a group of 18 bird species, 17 endemic to the Galapagos Islands and 1 inhabiting Cocos Island, belonging to Costa Rica (Galapagos Conservation Trust, 2023). These 18 species are categorized into 5 genera: *Geospiza*, *Camarrhynchus*, *Certhidea*, *Platyspiza*, and *Pinaroloxias* (Sato et al., 2001). Finches share traits such as size and color (Galapagos Conservation Trust, 2023), posing challenges for their morphological differentiation; however, distinctions exist among them, including habitats and diets, with the latter being closely linked to beak morphology (Villa et al., 2018). Furthermore, phylogenetic studies demonstrate that molecular differentiation is still challenging due to ongoing processes of hybridization between species, migration between islands, and their relatively short speciation time (Grant & Grant, 2021).

In studies concerning wildlife roadkill, a significant limitation arises when samples are morphologically unrecognizable, making species identification difficult or not possible. Molecular techniques offer a solution by enabling the identification of the affected species. DNA barcoding stands out as a widely used technique employed for species identification based on the detection of specific DNA markers that can differentiate among species/genera,

followed by comparison of the obtained sequence with genomic databases (Chen et al., 2023). The Cytochrome oxidase I marker (COI) is a mitochondrial marker highly utilized in evolutionary and phylogenetic studies due to its rapid evolution (Aliabadian et al., 2009), maternal inheritance, and highly conserved structure (Zhang et al., 2023). Based on that, COI region is frequently used in DNA barcoding since it allows not only species identification but other taxon identification. Another molecular marker highly used in different studies for species identification is Cytochrome B (cytB) (Farias et al., 2001).

Regarding finches' roadkill incidents, morphological identification may be challenging, and the use of the COI molecular marker could be limited due to the lack of COI region sequences for all finch species in genomic databases. Hence, it is necessary the search for a region that is acknowledged across all finch's species such as CytB region. However, CytB region utility in the study is constrained when attempting to identify closely related species, as is the case with finches.

The present study entails the development of a protocol for identifying birds killed by collisions that cannot be identified using traditional morphology-based methods on the road traversing San Cristóbal Island in the Galapagos Islands. DNA Barcoding was employed using universal primers Passer (Lohman et al., 2010) to amplify the COI sequence for family identification. In the case of finches, an additional primer based on the CytB sequence was utilized to distinguish between genera. This protocol enables comparison between amplified regions and genomic library databases in order to identify the affected species.

METHODS:

Sample collection:

Eight bird samples (J018, JPB018, CO32, J621, QD8, J032, CO17, CO01) were previously collected on the road of San Cristóbal Island that runs between the urban area of Puerto Baquerizo Moreno and Puerto Chino Beach.

These samples were preserved at the Galapagos Science Center in 90% ethanol at a temperature of -4ºC. Species identification based on morphology was performed for the 8 samples. This morphological identification is necessary for subsequent comparison with molecular identifications to validate the effectiveness of the designed protocol, after which they were sent to the Laboratorio de Biotecnología Vegetal de la Universidad San Francisco de Quito, where they were stored at -4ºC for further processing.

Pipeline design

For bird family identification a previously used primer was chosen. A set based on available literature on barcoding with birds of the order Passeriformes was chosen, namely PasserF1 and PasserR1. These primers bind to the COI (Cytochrome Oxidase I) region, amplifying a 648 bp segment.

For genera identification a set of primers was designed based on the CytB region since no available primer was found in literature. For the design of this primer set, 39 CytB sequences were extracted from NCBI of the following finch species: *Geospiza fortis*, *Geospiza fuliginosa*, *Camarhynchus parvulus*, *Camarhynchus pallidus*, *Certhidea fusca*, and *Platyspiza crassirostris*.

Using the CytB sequence of the specie *Platyspiza crassirostris* as reference, a primer set was designed with the aid of the primer designing tools available on NCBI (Primer BLAST) and Primer3. Subsequently, the primer sets were analyzed using OligoAnalyzer to identify secondary structures, followed by a BLAST analysis to confirm the identification of finch species. A primer set for finch genus identification was selected based on primer size,

ensuring it matched the desired product size, generated the fewest secondary structures with the lowest delta G, and exhibited a high percentage of identity with several of the reference sequences we utilized. The primer was designated as the PinzonF1 and PinzonR1 set.

DNA extraction

For DNA extraction, 25 milligrams of animal tissue were used as input material, employing the "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)" from DNeasy Blood and Tissue Kit following the manufacturer protocol with a modification in the elution step.

Following DNA extraction, quantification was conducted using a Nanodrop 1000 (Thermo Scientific), DNA integrity was analyzed through an agarose gel electrophoresis using a 1.5% agarose gel (100V, 30 minutes).

PCR: COI region

The universal primer set Passer were employed to amplify the mitochondrial COI region in the 8 morphologically identified samples. PCR amplifications were conducted in 25 µL reactions for each primer set, comprising concentration of 1X PCR Buffer, 1.5 mM MgCl2, 0.2 mM dNTPs mix, 0.2 of each primer, 1 µL of DNA, 0.2 U/µL of Platinum Taq DNA Polymerase. The thermal cycling conditions included an initial denaturation at 94ºC for 2 minutes, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 53ºC for 30 seconds, and extension at 72ºC for 1 minute, with a final extension step of 5 minutes at 72ºC. Subsequently, to verify the quality of PCR, agarose gel electrophoresis (1.5%, 100V, 30 minutes) was performed, and the amplicons were stored in a freezer at -4ºC.

Bioinformatics: COI region

8 amplicons from the PCR which amplified COI region were sent for Sanger sequencing to Macrogen Korea. After obtaining the sequencing results, the following bioinformatics pipeline was utilized to determain the species of each sample.

The pipeline uses the following softwares: Geneious Prime 2023.2.1 version and Mega 11. Protocol begin with Geneious prime; the REV sequence was converted to its reverse complement; after it both sequences were selected for trimming ends in order to cut low quality parts of the sequences. Subsequently, heterozygotes were located using "Find Heterozygotes." The FWD sequence was re-selected and marked as "Forward." Then, both sequences were chosen for de novo assembly to obtain a consensus sequence.

Sequences exported from Geneious Prime were imported into Mega software to correct nonspecific bases. Finally, within Mega, a BLAST was conducted to identify the Darwin finch species of the sample. These results were confirmed in Mega 11 by aligning the sample sequence with known CytB sequences of the identified species.

PCR: CytB region

With the previously mentioned steps, six samples were identified as Darwin finches (CO32, J621, QD8, J032, CO17, CO01), the same PCR protocol and thermal cycling were followed as mentioned above, with the difference being the use of specific primers designed for CytB region for finches: PinzonF1 and PinzonR1.

Bioinformatics: CytB region

The identification of the six finch samples (CO32, J621, QD8, J032, CO17, CO01) was performed using the previously mentioned bioinformatics pipeline. Additionally, a comparison of the obtained amplicon sequences with CytB sequences retrieved from NCBI was conducted to confirm the identification.

RESULTS:

DNA extraction

Table 1 displays the quantification values of the DNA extracted from the 8 samples. DNA concentration values range from 199.7 ng/µL to 1375.9 ng/µL. Quality indices for all 8 samples in the 260/280 index fall within the range of 1.8 to 2, except for sample QD8. Additionally, the 260/230 index shows values between 1.1 and 2.1 for all samples except QD8, indicating good quality of the extracted DNA. For subsequent PCRs, the samples were diluted to a concentration of 20 ng/µL.

Molecular identification of samples

Table 2 shows the results of the BLAST conducted on the sequences obtained from the Passer primer amplicons. It shows that primer Passer is able to separate between Thraupidae and Parulidae family. The identified species, query coverage, and percentage of identity are displayed, all of which exhibit high values ranging from 98 to 100%. It is confirmed that 2 out of the 8 samples belong to the species *Setophaga petechia*, while the other 6 samples are from Darwin finches. However, the precise genus of Darwin finch cannot be identified due to the absence of COI sequences in genomics libraries, with the BLAST only identifying the available finch sequences in NCBI genomic library (NCBI accession NC_039770; Liang et al., 2018) (NCBI KM891730; Lamichhaney et al., 2015).

Table 3 displays the results of the BLAST conducted on the sequences obtained from the specific primer for finches. Similar to Table 2, query values and percentage of identity are included, with both indices showing percentages close to 100%, ranging from 96 to 100%. It was confirmed that four of the samples indeed belonged to the genus *Geospiza*. However, there was discrepancy in the two samples initially identified based on morphology as *Platyspiza crassirostris*; the BLAST indicated that sample J621 corresponded to a finch from the *Certhidea* genus, and sample CO01 corresponded to a finch from the *Camarhynchus* genus.

DISCUSSION:

Amplification of the COI region efficiently enables differentiation between families, and as seen in the present results, it is possible to identify species if their COI region sequences are available on genomic libraries databases (Lijtmaer et al., 2012). In our protocol, a correct separation of families was achieved, as evidenced in Table 2, with a high percentage of identity and a high query coverage. Previous studies have utilized various primers amplifying the COI region for species identification (Chen et al., 2023), encompassing a broad spectrum of animals. It has been demonstrated that such studies yielded positive results, particularly in avian studies (Johnsen et al., 2010). Among the diverse bird families used in phylogenetic and bird identification studies are the Thraupidae family (Chaves et al., 2014) and the Parulidae family (Dove et al., 2008), which include Darwin finch's species and Setophaga petechia, respectively.

In the case of *Setophaga petechia*, identification can be achieved with a high percentage of identity as its COI region is well characterized and ample sequences are available in genomic libraries (Dove et al., 2008). This is due to extensive studies on this species owing to its wide distribution across the American continent (McAllister & Hnida, 2022). Despite the subspecies inhabiting the Galapagos no longer migrating to the continent, divergence from the continental subspecies occurred only 300,000 years ago, resulting in genetic sequences that remain highly similar (Chaves et al., 2012).

Nevertheless, concerning Darwin's finches, Table 2 demonstrates that all samples previously morphologically identified as finches have been identified as finches of the genus Geospiza. This is because only the COI region of two species of Darwin's finches from this genus has been sequenced (NCBI accession NC_039770; Liang et al., 2018) (NCBI KM891730; Lamichhaney et al., 2015). Due to these nonspecific results, it was necessary to amplify the cytochrome B region to achieve precise finch identifications.

The cytochrome B region has been previously used in species identification studies (Shen et al., 2013), although it is less commonly utilized than COI because the comprehensive

coverage of COI reference sequence databases, its variability, benefits in denoising protein-coding genes, and recent sequencing advancements support the standardization of COI for DNA barcoding studies (Andújar et al., 2018). These investigations encompass studies involving turtles (Shen et al., 2013), fish (Sevilla et al., 2007), or snakes (Laopichienpong et al., 2016); however, no studies were found that used CytB region for species or genera identification of birds of the Thraupidae family.

As demonstrated in Table 3, the use of the primer set Pinzon designed for this protocol efficiently identifies the genus *Geospiza*. However, in the two samples morphologically identified as belonging to the *Platyspiza* genus, there was discordance, since these samples were identified as part of to the *Certhidea* and *Camarhynchus* genera according to morphological identification, a second morphological identification was conducted by the Laboratorio de Zoología Terrestre from Universidad San Francisco de Quito based on coloration, size and shape of feathers, size and shape of beak or size and shape of legs, correcting the initial erroneous morphological identification, thus reinforcing the utility of our protocol as it can correct erroneous identification. Literature indicates challenges in identifying cryptic species or those in different age stages based solely on morphological identification (Krishnamurthy & Francis, 2012). Consequently, making morphological corrections holds significance in conservation plans as it enables precise data collection to enhance wildlife-related research studies (Gouda et al., 2020). Therefore, this protocol capable of conducting morphological corrections and utilizing unrecognizable or difficult to identify samples is highly valuable in order to understand roadkill in San Cristóbal and how it affects birds' population.

Regarding of the finches, there was discordance in identifying the exact species. This is because the use of molecular markers can be limited for species that are still closely related (Johnsen et al., 2010). For finches, movements within the islands (Beausoleil et al., 2022) and migrations between islands, hybridization processes among species (Grant & Grant, 2021), coupled with their short speciation time (Rands et al., 2013), hinder their molecular identification.

The protocol design in this study makes a significant contribution to conservation efforts in the Galapagos Islands. In the study conducted on Santa Cruz, one-tenth of the obtained samples were unusable due to the impossibility of morphological identification (García-Carrasco et al., 2020). Being able to expand the number of usable samples and make morphological corrections is crucial for conservation plans because having accurate data enables the implementation of efficient conservation strategies, especially in fragile ecosystems with protected areas like the Galapagos (Silva et al., 2020). This is important because incorrect morphological identification can potentially interfere with biodiversity conservation efforts, as undetected endangered endemic species may lack appropriate protection (Lohman et al., 2010). Furthermore, this protocol combines molecular identification processes with morphological identification.

Previous studies have demonstrated that a combination of both identification methods can yield better results. In the study conducted by Rodríguez et al. (2017), the combination of morphological and molecular identification allowed for the identification of 310% more samples than if only morphological identification had been used. The combination of both identification methods also brings benefits by promoting fast and reliable species identification. Just as corrections can be made to morphological identification based on molecular identification, morphological identification can also correct erroneous molecular identifications in closely related species (Klippel et al., 2015), such as finches.

CONCLUSIONS:

These results demonstrate that the proposed protocol can identify bird families and species based on the COI region, such as *Setophaga petechia*. Regarding Darwin finches, the primer designed for this protocol, which amplifies the cytochrome B region, efficiently identifies genera from Darwin's finches. It is noteworthy that the protocol can potentially correct erroneous morphological identifications and utilize unrecognizable or badly conserved samples for morphological identification, thereby providing additional information for baseline data used in conservation plans in the Galapagos Islands. Lastly, it's worth mentioning that this protocol is not only applicable to San Cristobal Island but can also be utilized on other islands within the archipelago.

TABLES:

Table 1. DNA quantification and quality indices (260/280 and 260/230) of the 8 bird samples.

Sample ID	Concentration (ng/uL)	260/280	260/230
JPB018	582.9	1.83	1.33
J018	706.3	1.87	1.62
CO17	473.1	1.92	1.59
CO32	199.7	1.87	1.15
QD8	204.0	1.69	0.58
J032	933.0	1.93	1.97
J621	1375.9	1.98	2.07
C001	1027.1	1.89	1.76

Description: Table 1 displays the DNA concentration of the 8 samples, as well as the quality indices 260/280 and 260/230. Concentrations range from 199 to 1375 ng/uL. Quality indices range from 1.69 to 1.98 for 260/280 index and from 0.58 to 2.07 for 260/230 index.

Table 2. BLAST results from COI sequences amplified using primer Passer and the comparison with the morphological identification.

Sample ID	Morphological identification	Query coverage	Identity percentage	Molecular identification	Family
J018	*S. petechia*	99%	98.50%	*S.petechia*	Parulidae
		99%	98.65	*S.petechia*	
JPB018	*S. petechia*	100%	98.51%	*S.petechia*	
		99%	98.80%	*S.petechia*	
JO32	*G. fuliginosa*	99%	99.70%	*G.fortis*	Thraupidae
		99%	99.50%	*G.magnirostris*	
CO32	*G.fuliginosa/fortis*	99%	99.55%	*G.fortis*	
		99%	99.41%	*G.magnirostris*	
QD8	*G. fortis*	99%	99.85%	*G.fortis*	
		99%	99.70%	*G.magnirostris*	
CO17	*G.fuliginosa*	100%	99.26%	*G.fortis*	
		100%	99.12%	*G.magnirostris*	
J621	*P. crassirostris*	99%	95.95%	*G.fortis*	
		99%	95.95%	*G.magnirostris*	
C001	*P. crassirostris*	100%	98.98%	*G.fortis*	
		100%	98.83%	*G.magnirostris*	

Description: Table 2 shows the results of molecular identification by BLAST (top 2 primer hits) of the amplicons obtained from the Passer primer PCR and their comparison with morphological identification. It can be observed that both morphological identifications of *Setophaga petechia* coincide with molecular identification with a high percentage of identity. The 6 morphological identifications of finches, 2 from the *Platyspiza* genus and 4 from the *Geospiza* genus, are identified as belonging to the *Geospiza* genus.

Table 3. BLAST results from CytB sequences amplified using primer Pinzon used for finches and the comparison with the morphological identification.

Sample ID	Morphological identification	Query coverage	Identity percentage	Molecular identification
JO32	*G. fuliginosa*	96%	99.33%	*G.fortis*
		96%	99.33%	*G.magnirostris*
CO32	*G.fuliginosa/fortis*	99%	99.77%	*G.fortis*
		99%	99.77%	*G.fuliginosa*
QD8	*G. fortis*	99%	99.89%	*G.magnirostris*
		99%	99.89%	*G.cornirostris*
CO17	*G.fuliginosa*	99%	99.54%	*G.fuliginosa*
		99%	99.43%	*G.scandens*
J621	*P. crassirostris*	99%	99.52%	*C. fusca*
		97%	100%	*C. fusca*
C001	*P. crassirostris*	97%	99.98%	*C.parvulus*
		97%	99.89%	*C.pallidus*

Description: Table 3 presents the results of molecular identification by BLAST (the top 2 hits) of the amplicons obtained from the PCR with the specific Pinzon primer for the CytB region of finches. It is observed that in the 4 species morphologically identified as belonging to the *Geospiza* genus, molecular identification matches the morphological identification; however, there are variations in species identification. In the case of the 2 samples morphologically identified as belonging to the *Platyspiza* genus, there was discordance as one was identified as belonging to the *Camarhynchus* genus and the other sample was identified as belonging to the *Certhidea* genus.

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