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AMANDA ALVES DE MELO

MESMA INFORMAÇÃO, NOVAS APLICAÇÕES: REVISITANDO *PRIMERS* DO GENE COI EM AVES E MELHORANDO A IDENTIFICAÇÃO POR *DNA BARCODING*

Orientadora: Dra. Mariana Pires de Campos Telles
Co-orientador: Dr. Rhewter Nunes

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AMANDA ALVES DE MELO

MESMA INFORMAÇÃO, NOVAS APLICAÇÕES: REVISITANDO *PRIMERS* DO GENE COI EM AVES E MELHORANDO A IDENTIFICAÇÃO POR *DNA BARCODING*

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Orientadora: Dra. Mariana Pires de Campos Telles
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Ata nº **88** da sessão de Defesa de Dissertação de **Amanda Alves de Melo**, que confere o título de Mestre(a) em **Genética e Biologia Molecular**, na área de concentração em **Genética e Biologia Molecular**.

Ao/s **trinta e um dias do mês de março de dois mil e vinte e um**, a partir da(s) **14h00**, por videoconferência, seguindo portaria CAPES no. 36 de 16 de março de 2020 e recomendação da UFG, realizou-se a sessão pública de Defesa de Dissertação intitulada **"Mesma informação, novas aplicações: Revisitando primers do gene COI em aves e melhorando a identificação por DNA barcoding"**. Os trabalhos foram instalados pelo(a) Orientador(a), Professor(a) Doutor(a) **Mariana Pires de Campos Telles (ICB/UFG)** com a participação dos demais membros da Banca Examinadora: Professor(a) Doutor(a) **Dra. Cíntia Pelegrineti Targueta (UFG)**, membro titular externo; Doutor(a) **Ramilla dos Santos Braga**, membro titular externo. Durante a arguição os membros da banca **não fizeram** sugestão de alteração do título do trabalho (abaixo). A Banca Examinadora reuniu-se em sessão secreta a fim de concluir o julgamento da Dissertação, tendo sido(a) o(a) candidato(a) **APROVADA** pelos seus membros. Proclamados os resultados pelo(a) Professor(a) Doutor(a) **Mariana Pires de Campos Telles**, Presidente da Banca Examinadora, foram encerrados os trabalhos e, para constar, lavrou-se a presente ata que é assinada pelos Membros da Banca Examinadora, ao(s) **trinta e um dias do mês de março de dois mil e vinte e um**.

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Resumo

A identificação de espécies se dá através da comparação de características morfológicas, bioquímicas ou genéticas. Todas as áreas de conhecimento para a delimitação de espécies estão conectadas e devem ser analisadas de formas complementares para preencher lacunas de informação existentes em cada área. O processo de identificação molecular de espécies por meio da comparação da informação genética denominado DNA *barcode* consiste na análise da sequência de nucleotídeos de uma determinada região para comparação das diferenças entre as espécies. Na maior parte dos animais, incluindo o grupo das aves, o gene citocromo c oxidase I (COI) é amplamente utilizado para esta finalidade e na maior parte dos casos, apresenta maior variação entre espécies do que a variação dentro dos indivíduos da mesma espécie e fornece informações elucidativas sobre a diferenciação das espécies. A escolha dos *primers* para uma aplicação de DNA *barcode* é uma etapa crucial pois depende do objetivo do estudo e do grupo taxonômico em questão. Análises *in silico* de *primers* podem avaliar suas propriedades físico-químicas, o número de espécies alcançadas por eles e então direcionar a escolha dos melhores *primers* para diferentes objetivos de pesquisa, economizando tempo e dinheiro. Desta forma, este presente trabalho buscou responder algumas perguntas relacionadas à eficiência dos *primers* disponíveis para o gene COI em aves e às sequências genômicas mitocondriais de aves disponíveis em bancos de dados: i) Qual a abrangência taxonômica (número de espécies) alcançada pelos *primers* do gene COI em aves? Os *primers* dito como universais conseguem cobrir realmente todas as espécies?; ii) Quais as ordens de aves que possuem mais sequências genômicas mitocondriais disponíveis nos bancos de dados e qual a significância da representação destas sequências dentro do número total de espécies das ordens?; iii) Quais *primers* apresentam as condições físico-químicas ideais para aumentar as chances de uma amplificação de sucesso em experimentos laboratoriais? e; iv) Quais os conjuntos de *primers* são os mais apropriados para garantir a cobertura total das espécies de aves?

Palavras-chave

COI; tráfico; conservação; DNA barcode; forense; *in silico*; PCR

Abstract

Molecular identification of species occurs through the comparison of either morphological, biochemical or genetic characteristics. All of these areas of knowledge for delimiting species are connected and should be analyzed as a complement to fill gaps of information of each area. The process of molecular identification of species through comparison of genetic information named DNA barcode consists of analyzing nucleotide sequences of a certain region to compare the differences between species. On the majority of the animals, including birds, the cytochrome c oxidase I (COI) gene is widely used for this purpose and in most cases, it shows the ideal greater variation in its sequence among species rather than within individuals of the same species, providing elucidative information about species differentiation. Choosing primers for a DNA barcode application is a crucial step because it depends on the aim of the study and on the taxonomic group of interest. Primers in silico analysis can evaluate its physicochemical properties, the number of species reached by them and then direct the choice of the best primers for the different research objectives, saving time and money resources. Therefore, this present work aimed to answer questions related to the efficiency of the available primers for the avian COI gene and to the avian mitochondrial genome sequences available in databases: i) What is the taxonomic coverage (number of species) reached by the avian COI gene primers? Are the universal primers actually capable of covering all species?; ii) Which avian orders have the most mitochondrial genome sequences available on the databases and what is its representativeness significance within the total number of species for each order?; iii) Which primers show the ideal physicochemical properties to increase the chances of successful amplification in laboratorial experiments? and; iv) Which primer sets are the most suited to guarantee full species recovery within the avian group?

Keywords
COI; traffic; conservation; DNA barcode; forensics; in silico; PCR

Introdução

A identificação de espécies, baseando-se no seu conceito biológico que consiste em indivíduos que se reproduzem naturalmente gerando indivíduos viáveis e férteis e que compartilham das mesmas características (DE QUEIROZ, 2007), sejam elas morfológicas (MIRANDA et al., 2018), bioquímicas ou genéticas (PARISI DUTRA et al., 2017), se dá através da comparação destes caracteres. Esta comparação das características é feita através de marcadores, que dentro da delimitação de espécies, são condições dos indivíduos que os permitem ser reunidos em grupos de condições similares.

Os marcadores clássicos para delimitação de espécies envolvem características morfológicas como medidas de estruturas anatômicas (BREITMAN et al., 2018) e análise do padrão de vocalização no caso de animais que emitem sons (OVASKAINEN; MOLITERNO DE CAMARGO; SOMERVUO, 2018). Marcadores químicos e bioquímicos trazem a avaliação do perfil de produção proteica característico a cada espécie permitindo a sua diferenciação (FARMER et al., 1985). E os marcadores moleculares que envolvem a comparação da constituição do material genético das espécies, sejam elas o número, o tamanho e o tipo dos cromossomos (TARGUETA et al., 2021) ou a composição nucleotídica de algumas regiões do genoma das espécies (BITANYI et al., 2011). Todas estas áreas de conhecimento para a delimitação de espécies estão conectadas e devem ser analisadas de formas complementares para evitar viés e preencher lacunas de informação existentes para cada área (HORTAL et al., 2015).

Buscando complementar as lacunas e as incertezas existentes na classificação e no acesso à biodiversidade, o processo de identificação molecular de espécies por meio da comparação da informação genética surgiu por volta de 1980 como uma ferramenta útil para auxiliar nesses processos (DESALLE; GOLDSTEIN, 2019). Entretanto, somente em 2003 que a ferramenta de identificação molecular pela comparação de regiões de DNA recebeu formalmente a denominação, o termo DNA *barcode*, e teve seu uso disseminado e ampliado (HEBERT et al., 2003). A ferramenta consiste na análise da sequência de nucleotídeos de uma determinada região para comparação das diferenças entre as espécies, de forma que cada espécie tenha um conjunto de sequências específicas com pequenas variações entre seus indivíduos (HEBERT et al., 2003).

A região padrão a ser utilizada como marcador de DNA *barcode* é variável para cada grupo taxonômico, pois cada conjunto de organismos apresenta diferentes modos de reprodução, tempo de geração e consequentemente, diferentes taxas de evolução do seu material genético (CLARE et al., 2008; HEBERT et al., 2003). Em plantas, genes codificadores de proteínas do genoma cloroplastidial são os que apresentam maior poder de discriminação das espécies, principalmente quando utilizados de forma combinada (BURGESS et al., 2011). Já no grupo dos animais, o genoma mitocondrial possui maior taxa de mutação em relação ao genoma nuclear e por isso, acarreta em maior divergência entre as sequências de suas espécies ao longo do tempo, principalmente por possuir características como ausência de sistema de reparo da replicação e baixa taxa de recombinação (ALLIO et al., 2017).

Na maior parte dos animais, incluindo o grupo das aves, o gene citocromo c oxidase I (COI), com aproximadamente 1.500pb, é um grande protagonista na técnica de DNA *barcode* por possuir diferenças nucleotídicas suficientes para discriminar espécies (HEBERT; RATNASINGHAM; DEWAARD, 2003), sendo inclusive recomendado como a região universal para identificação molecular em animais (HEBERT et al., 2004; KERR et al., 2007). Dentro das aves, é um gene com uma das mais baixas taxas de evolução molecular (LAVINIA et al., 2016; SACCONE et al., 1999) e por isso, consegue fornecer entendimentos mais profundos das relações filogenéticas, principalmente de espécies com história de divergência mais antiga (HEBERT et al., 2003).

Independente da região a ser utilizada, para uma região ser um bom marcador de DNA *barcode*, ela precisa apresentar maior variação nas suas sequências entre espécies (interespecíficas) do que variações dentro dos indivíduos da mesma espécie (intraespecífica) (HEBERT et al., 2003). Esta diferença nas variações deve ser significativa e medida pela diferença das médias par-a-par das distâncias das sequências entre os indivíduos de espécies diferentes e entre indivíduos da mesma espécie (HEBERT et al., 2004), o chamado *barcoding gap*. Observar essa diferença entre as variações inter- e intraespecíficas é de extrema importância para confiar nos resultados obtidos por DNA *barcode*, pois somente com esta diferença significativa é que é possível ter informações suficientes e esclarecedoras sobre a divergência molecular das espécies (ČANDEK; KUNTNER, 2015; HEBERT et al., 2004).

A técnica de DNA *barcode* vem sendo amplamente utilizada desde a sua popularização em 2003, não somente para auxiliar na resolução das incertezas taxonômicas, como foi sua proposta inicial (HEBERT et al., 2003) mas para diversas outras finalidades como combate ao tráfico ilegal (GONÇALVES et al., 2015), combate a crimes contra o consumidor (NEWMASTER et al., 2013), identificação de carcaças animais (KLIPPEL et al., 2015), identificação de presas da dieta de animais (HEIM et al., 2019), dentre muitos outros. Em muitas dessas aplicações, existe a necessidade de recuperar um grande número de espécies e em alguns casos, a qualidade da amostra analisada nem sempre é alta, como é o caso das análises forenses e de *metabarcoding*. Para isso, é importante utilizar-se de *primers* universais, que são pensados para se complementar em regiões conservadas entre as espécies alvo de um determinado grupo taxonômico, visando recuperar o maior número de táxons possíveis (LOPEZ-OCEJA et al., 2016).

A escolha dos *primers* para uma aplicação de DNA *barcode* é uma etapa crucial pois depende do objetivo do estudo e do grupo taxonômico em questão (FICETOLA et al., 2010). Uma etapa de análise de *primers* para auxiliar na escolha dos melhores conjuntos é a avaliação por PCR *in silico*, ou seja, por simulações computacionais que buscam no conjunto de sequências desejadas, quais as sequências que possuem complementariedade com os *primers* analisados (FICETOLA et al., 2010). Algumas ferramentas existentes para realizar estas análises *in silico* avaliam as propriedades físico-químicas dos *primers* individuais e com seus possíveis pares, assim como avaliam o poder de cobertura taxonômica, ou seja, o número de espécies alcançadas pelos *primers* na simulação computacional (KREER et al., 2020). Além disso, esta avaliação prévia permite o direcionamento preciso da escolha dos *primers* e economiza não somente tempo mas recursos financeiros para os pesquisadores.

Estas avaliações *in silico* são possíveis pois hoje existe um grande número de sequências *barcode* disponíveis em bancos de dados públicos oriundas das diversas aplicações da técnica (RATNASINGHAM; HEBERT, 2007). Além das sequências *barcode*, a disponibilização das sequências genômicas, como o genoma mitocondrial dos animais, pode ser útil para diversos tipos de análises bioinformáticas *in silico*, incluindo a avaliação e desenho de *primers* para DNA *barcode* (ELBRECHT; LEESE, 2017; FICETOLA et al., 2010; KREER et al., 2020). Entretanto, em grupos muito diversos como o das aves, o status do número e a representatividade das sequências genômicas disponíveis nos bancos de dados para este grupo ainda é desconhecido.

Uma outra informação também ainda não conhecida, é a quantidade, a eficiência e a abrangência taxonômica alcançada pelos *primers* desenvolvidos para identificação por DNA *barcode* dentro do grupo das aves, um grupo que possui muitos estudos desta técnica para diversas aplicações (DESALLE; GOLDSTEIN, 2019). Utilizar as sequências genômicas disponíveis em bancos de dados para avaliar os *primers* também disponíveis para o gene COI, a principal região utilizada na identificação por DNA *barcode* em aves, é importante para entender o comportamento e a eficiência destes *primers* no grupo e para auxiliar na escolha dos melhores *primers* para os diferentes objetivos de pesquisa. Projetos que buscam recuperar grande número de espécies devem preferir *primers* universais que realmente sejam complementares para o maior número de espécies, diferente dos projetos que são específicos à um grupo taxonômico que não possuem essa necessidade.

Desta forma, este presente trabalho buscou responder algumas perguntas relacionadas à disponibilidade dos *primers* para o gene COI em aves e às sequências genômicas mitocondriais de aves disponíveis em bancos de dados: i) Qual a abrangência taxonômica (número de espécies) alcançada pelos *primers* do gene COI em aves? Os *primers* dito como universais conseguem cobrir realmente todas as espécies?; ii) Quais as ordens de aves que possuem mais sequências genômicas mitocondriais disponíveis nos bancos de dados e qual a significância da representação destas sequências dentro do número total de espécies da ordem?; iii) Quais *primers* apresentam as condições físico-químicas ideais para aumentar as chances de uma amplificação de sucesso em experimentos laboratoriais? e; iv) Quais os conjuntos de *primers* são os mais apropriados para garantir a cobertura total das espécies de aves?

Objetivos

Objetivo geral

O objetivo geral deste trabalho foi identificar e avaliar os melhores *primers* utilizados na identificação molecular de aves utilizando o gene COI, tanto no aspecto químico como no da abrangência taxonômica, de forma a contribuir com a melhora na identificação de espécies por *DNA barcoding* ao direcionar a escolha dos melhores *primers* para cada objetivo de pesquisa.

Objetivos específicos

- Avaliar a disponibilidade e a representatividade das sequências mitocondriais completas para cada ordem do grupo das aves;
- Recuperar os *primers* disponíveis que estão sendo atualmente usados na identificação molecular de aves para acessar o gene COI como marcador de DNA *barcode;*
- Avaliar a capacidade de cobertura taxonômica, propriedades físico-químicas e performance dos *primers* recuperados com o uso de PCR *in silico*;
- Fornecer os melhores conjuntos de *primers* para identificação molecular de aves no aspecto amplo da classe ou específico para cada ordem.

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Same information, new applications: Revisiting primers for the avian COI gene and improving DNA barcoding identification

Authors:
Amanda Alves de Melo[1]; Rhewter Nunes[2]; Mariana Pires de Campos Telles[1,3]

Affiliations:
[1]Programa de Pós-Graduação em Genética e Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia – Goiás, Brazil.
[2]Laboratório de Genética & Biodiversidade, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia – Goiás, Brazil.
[3]Escola de Ciências Agrárias e Biológicas, Pontifícia Universidade Católica de Goiás, Goiânia – Goiás, Brazil.

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Correspondence:
Dr. Mariana Pires de Campos Telles
Laboratório de Genética & Biodiversidade, Departamento de Genética, Instituto de Ciências Biológicas, Universidade Federal de Goiás – GO – Brasil
tellesmpc@gmail.com

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Data availability statement

The primers' sequence analyzed in this current study were retrieved from previously published papers indexed in the Web of Science Database available at https://clarivate.com/webofsciencegroup/solutions/web-of-science/ (See Supplementary Table 1 for further reference information). The mitochondrial genome sequences datasets analyzed in this study were retrieved from the Nucleotide database from the GenBank sequence archive of the National Center for Biotechnology Information (NCBI), available at https://www.ncbi.nlm.nih.gov/ (See Supplementary Table 3 for GenBank Accession numbers).

Abstract:

The process of molecular identification of species known as DNA barcoding consists on discriminating taxa based on cumulative differences of their DNA sequences and is widely used within animals, including birds. Finding the best genomic resources, such as primers, to reach most of the species within the studied groups and evaluating the coverage breadth and physicochemical properties of primers before testing in the laboratory can save time and financial resources. Therefore, this work aimed to retrieve the available primers for the COI gene currently used on the molecular identification of birds, evaluate them for its coverage range, physicochemical properties, and performance on in silico PCR. Afterwards, we provide the best primer subsets to cover the highest number of avian sequences and the best individual primers for each bird order in terms of coverage breadth. Thirty-one bird orders had at least one COI sequence to serve as template, 156 primers available for assessing the COI gene were evaluated and 118 could bind to at least one template sequence. No primer subset alone could cover all template sequences, however, when combining two subsets, the complete coverage of avian COI sequences analyzed was achieved. We were able to visualize the individual and joint coverage range, the physicochemical properties and PCR performance of the primers designed for accessing the avian COI gene. Also, we provide the optimal primer subsets for more specific or broad objectives in order to guide researchers in the process of choosing the best primer set for each individual goal.

1. Introduction

The process of molecular identification of species using DNA consists on discriminating taxa based on the cumulative differences of their DNA sequences (Pereira et al. 2008). It began to help and complement species' classic identification through morphology (Hebert et al. 2004). This technique has been used since the 1980s, but it has been formally identified only in 2003 (DeSalle and Goldstein 2019). Three publications named the molecular identification method using DNA sequences as DNA barcoding (Hebert, Cywinska, et al. 2003; Hebert, Ratnasingham, et al. 2003; Stoeckle 2003) and globalized its application. Ever since its formalization, the number of papers about DNA barcoding has only risen to 2016. It has been used for specimens or species identification applied to many areas, such as forensic, taxonomy, and conservation (DeSalle and Goldstein 2019).

Among animals, DNA barcoding is used to complement classic taxonomic analysis on biodiversity assessment (Weigt et al. 2012), on cryptic species identification (Hawlitschek et al. 2011), on forensic research (Gonçalves et al. 2015) and on environmental (eDNA) studies, which try to recover all the living organisms of an environment such as soil or animal scats using metabarcoding (Berry et al. 2017; Epp et al. 2012). Mitochondrial DNA has some features that have made this the most used molecular marker on the identification of animal species, such as the absence of introns, maternal inheritance, and faster evolutionary rate when compared to nuclear DNA (Bernt et al. 2013; Brown et al. 1979; Saccone et al. 1999).

For vertebrates, mitochondrial genome shows absolute values of mutation rate higher than its nuclear analogous (Allio et al. 2017), which winds up increasing sequence divergence more rapidly over time mainly due to its lack or absence of a replication repair system and low recombination rate (Allio et al. 2017; Bernt et al. 2013). Within birds, the cytochrome c oxidase I (COI) gene is considered one of the slowest evolving mitochondrial genes (Lavinia et al. 2016; Saccone et al. 1999), and therefore, it is more likely to provide deep phylogenetic insights than other mitochondrial genes (Hebert, Cywinska, et al. 2003). The COI gene has proven to successfully discriminate congeneric species within the animal phyla due to enough sequence divergence (Hebert, Ratnasingham, et al. 2003) and for this reason, it is widely used as the standard barcode region for the molecular identification of species of many animal groups (Hebert et al. 2004; Kerr et al. 2007).

The technique of DNA barcoding is a valuable tool for helping to solve crimes against wildlife and for understanding patterns of genetic structure and divergence among taxa, including birds (Kerr et al. 2009; Tavares et al. 2011). To achieve these goals, there is a great demand for genomic resources, such as primers, to reach most of the species within the studied groups (Coghlan et al. 2012). Designing or selecting existing primers is crucial to guarantee a balance between high specificity and high amplification efficiency to answer all the questions of a molecular identification study (Dieffenbach et al. 1993; Vamos et al. 2017). Therefore, evaluating the coverage breadth and physicochemical properties of primers on an in-silico approach before testing in the laboratory can be essential for saving time and financial resources (Elbrecht and Leese 2017). In this context, the primers' status and efficiency for the COI gene used for molecular identification of birds and its coverage breadth within this group remain unknown.

Therefore, this work aimed to retrieve the available primers for the COI gene currently used on the molecular identification of birds, evaluate them for its coverage range, physicochemical properties, and performance on in silico PCR. Afterwards, we provide the best primer subsets to cover the highest number of avian sequences and the best individual primers for each bird order in terms of coverage breadth. In addition, we also analyzed the record availability of complete mitochondrial genomes for every bird order.

2. Methods

Data retrieval

We retrieved scientific papers available on the Web of Science database using the following keywords: “Metabarcoding”, “DNA barcoding”, “barcoding”, “birds”, “avian”, and “avifauna” + name of all the 36 bird orders according to NCBI Taxonomy. Only full papers with DNA barcode and metabarcoding applications on birds published until May 2020 were selected. A deep search for the primers used on the identification of birds on the selected papers was performed. Both the primer’s name and its sequence were organized into a table with their proper original references listed (Supplementary Table 1).

To retrieve complete COI sequences for the following primer evaluation analysis, all bird species with complete or partial annotated mitochondrial genome available up to July 20th 2020, on the National Center for Biotechnology Information (NCBI) were used as templates. We used the function *esearch* from the “reutils” package in R (Scholf 2016) to locate all NCBI entries of avian mitogenomes using the search term “Order name [orgn] and complete genome [title]” on the "nuccore" database. The accession number of all these entries were retrieved using the *efetch* function of the same R package with "acc" as the retrieval type, and "text" as the data mode of the records returned. From the retrieved accession numbers, COI sequences were extracted using the "AnnotationBustR" package (Borstein and O'Meara 2018) and written as fasta files.

In silico primer evaluation

To evaluate the primers coverage range and compute its PCR-relate physicochemical properties, we used the R package "openPrimeR" (Kreer et al. 2020) (available at: https://github.com/matdoering/openPrimeR-User). For the evaluation step, every bird order was considered a group, and its species sequences were template sequences for their group. The allowed target binding region for primer evaluation was the program's default first, and the last 30bp for forward and reverse primers, respectively. However, off-target binding was allowed to evaluate for binding events on the full length of the COI gene. The maximum number of allowed mismatches between a primer and a template was seven base pairs. For a group to be considered covered by a primer, the minimum required number of covered template sequences per primer was one. Primers were evaluated for allowing and forbidding mismatches on the last seven bases of the 3' end of the primer sequence and for primer lengths of 18-26 and 18-30pb. The different primer lengths here tested resulted in the same number of primers with binding events, and allowing mismatches on the 3' end of the primers did not result in a significant increase of this same result (data not shown). Therefore, we continued the following analysis only with the results of primers ranging from 18-30bp with mismatches on the last seven bases of the 3' end forbidden, since the latter setting is widely recommended to increase assurance of good PCR amplification results (Dieffenbach et al. 1993).

The evaluated primers were then filtered to select the ones who fulfilled all the input and coverage constraints, as well as the PCR conditions. The remaining properties were set as the package's default settings (Supplementary Table 2). The ten forward and reverse primers that could cover the highest number of template sequences, and all the primers that passed on the filtering phase were submitted to a second evaluation step to sample primers subsets that, when combined, sequence coverage is maximized. Coverage statistics of the evaluated primers were performed within "openPrimeR", and the graphics of this analysis were created using the "ggplot2" package in R (Wickham 2016).

3. Results

Data retrieval

A total of 341 papers were retrieved, of which 238 were not considered on the following analysis because they either did not use the COI gene for molecular identification of birds or identified only species related to birds on their barcoding and metabarcoding analysis. From the 103 remaining papers, it was possible to identify 192 primers for the COI gene from 56 different references (Supplementary Table 1) with sequence length varying from 17 to 30bp (mean=22.89bp). Ninety-one primers had forward orientation and 101 reverse. The year of publication of these primers ranged from 1994 to 2020, with 2017 being the year with most publications of novel primers for accessing the COI barcode region (n=30) (Figure 1A).

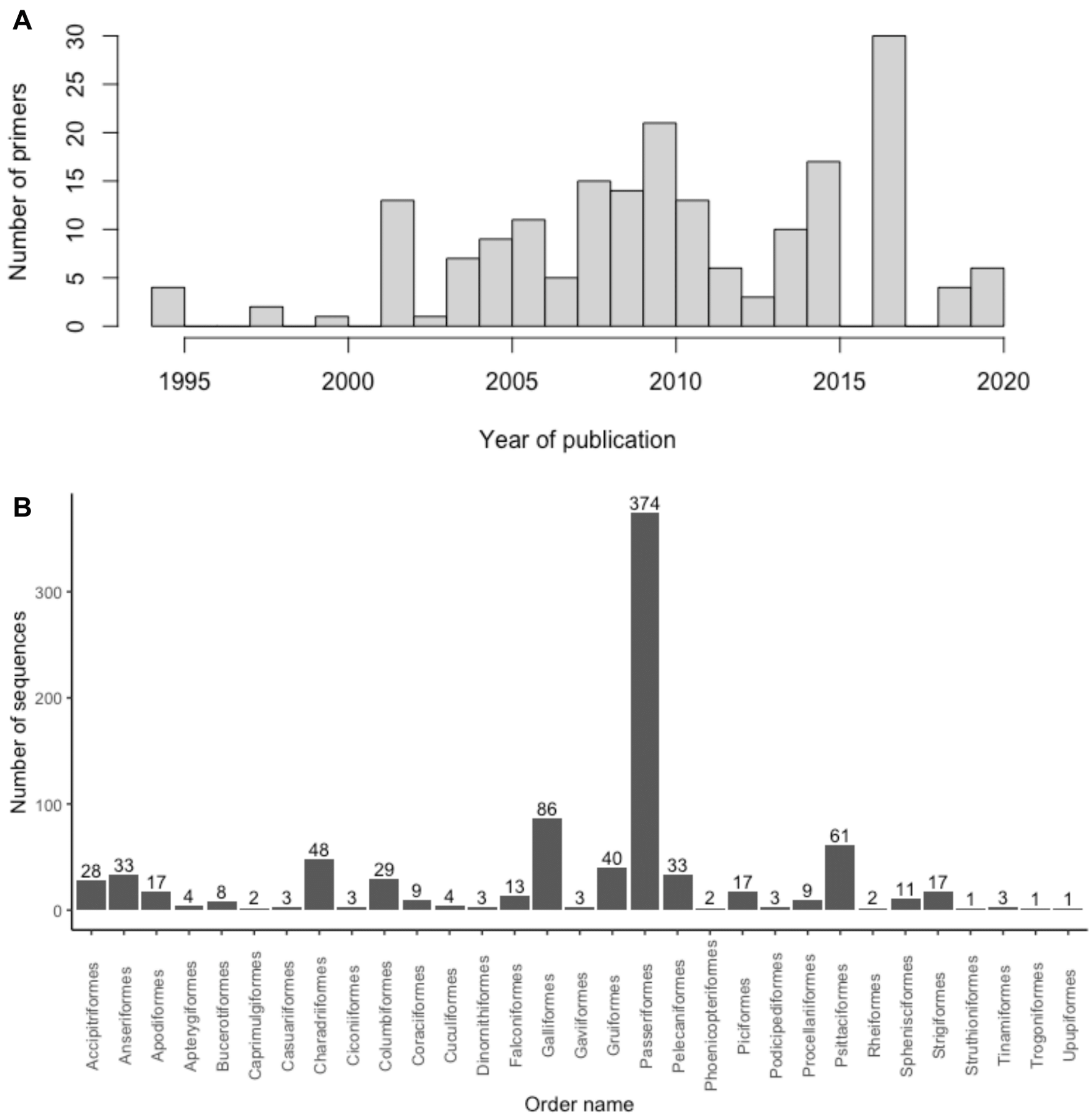

Fig. 1 Retrieved data distribution. A) Distribution of published primers for the barcode region of the avian COI gene throughout the years. B) Number of complete COI sequences available for each bird order retrieved from complete or partial annotated mitochondrial genomes available on the NCBI database.

All these primers were used 474 times in distinct set combinations, 240 times for forwarding primers, and 234 times for reverse. The forward primer most frequently used was BirdF1 (n=73, 30.42%) (Hebert et al. 2004), followed by AWCF1 (n=10, 4.17%) (Patel et al. 2010) and LTyr (n=9, 3.75%) (Tavares and Baker 2008). On the other hand, the reverse primers most frequently used were BirdR1 (n=37, 15.81%) and BirdR2 (n=24, 10.26%) (Hebert et al. 2004), followed by COIbirdR2 (n=12, 5.13%) (Kerr et al. 2009). There was a significant negative relation between the primer's frequency of usage and its year of publication (r= -0.17, p=0.01), meaning that the older the year of design and publication of a primer, the higher its frequency of usage (Supplementary Figure 1). Of the 192 retrieved primers, only 156 were included in the following analysis because six had IUPAC codes not recognized by the used evaluation tool. The other 30 had elevated levels of degeneracy (>30) in which the great number of possible base combinations caused the program to crash, and therefore, were excluded in the following steps.

Thirty-one bird orders had at least one representative with their complete or partial annotated mitochondrial genome available at NCBI and could have complete COI sequences retrieved. The orders with no mitochondrial genomic resources available were Cariamiformes, Coliiformes, Galbuliformes, Musophagiformes, and Opisthocomiformes, and, therefore, were not included in this study. Eight hundred sixty-eight complete COI sequences were retrieved (Supplementary Table 3) and were used as template sequences for each bird order, with Passeriformes having the greatest number of sequences available (n=374, 43.08%) and Struthioniformes, Trogoniformes, and Upupiformes the fewest number (n=1, 0.11% each) (Figure 1B).

In silico primer evaluation

Primer coverage

Six sets of primers were found to be redundant, that is, had different names but had the same sequence and therefore bound to the same region (VertebrateR1 & VR1_t1; AR & BirdR4; AF & BirdF4; BirdH_351d_370d_R & Bird-H351d_R; CO1R & COXR; CO1F & COXF).

With up to seven primer-template mismatches allowed, a total of 118 primers (75.64%) had at least one binding event, that is, could bind to at least one template sequence. Of the 38 primers that did not bind to any template sequences, forward primers were the most common among them (n=23). The five primers that could cover the highest number of sequences were: mCOIR2 (n=767, 88.36%), K_Bird_F1 (n=761, 87.67%), the redundant set VertebrateR1 and VR1(n=718, 82.72% each), AWCintR4 (n=706, 81.34%), and BirdF1d (n=688, 79.26%) (Table 1). On the other hand, the five primers that could cover the lowest number of sequences were: AWCintF2, L6615(tTyr)_COI_F, Pel-F2-COI, Schutz03F, and BC_392F (n=1, 0.12% each). There was a positive but not significant relation between the primer's degeneracy level and its coverage ratio (r=0.11, p=0.15).

Table 1. Top 5 primers (overall, forward, and reverse) with the highest number of covered template sequences. Every template sequence covered by a primer is considered a primer-template binding event. NO and N refer to the number of avian orders and the number of template sequences covered by the primer, respectively. %: percentage of template sequences covered by the primer.

Top 5 primers				Top 5 forward primers				Top 5 reverse primers			
Primer name	**NO**	**N**	**%**	**Primer name**	**NO**	**N**	**%**	**Primer name**	**NO**	**N**	**%**
mCOIR2	30	767	88.36	K_Bird_F1	27	761	87.67	mCOIR2	30	767	88.36
K_Bird_F1	27	761	87.67	BirdF1d	31	688	79.26	VertebrateR1 & VR1	30	718	82.72
VertebrateR1 & VR1	30	718	82.72	BirdHRM-F	27	688	79.26	AWCintR4	31	706	81.34
AWCintR4	31	706	81.34	AWCintF3	25	687	79.15	CO1R	31	680	78.34
BirdF1d	31	688	79.26	AvMiF1	24	622	71.66	BirdR1dt	27	636	73.27

Not all primers with the highest number of binding events were capable of binding to the template sequences of all avian orders (Table 1). Only three primers were capable of binding to at least one template sequence of all bird orders: AWCintR4, BirdF1d, and CO1R (Table 1). Seven primers could bind to template sequences of only one avian order: COIaRt_F, PsEmpCOIF, L6615_(tTyr)_COI, AWCintF2, PelF2COI, Schutz03F, and BC_392F, with only the first two binding to more than one template sequences (Supplementary Table 4).

Ciconiiformes was the order with the highest mean of primer coverage (49), in other words, the order that had the highest mean of different primers bound to its template sequences, followed by Podicipediformes (40.33), Cuculiformes (38), Falconiformes (36.1) and Apodiformes (35.41) (Figure 2). On the opposite side, the orders that had the lowest mean of primer coverage were Rheiformes (20), Trogoniformes (21), Struthioniformes (25), Bucerotiformes (27.13), and Accipitriformes (27.57). However, the orders with the most variable number of primers bound to its template sequences were Psittaciformes, Passeriformes, and Piciformes (Figure 2). There was no significant relation between the number of analyzed template sequences and the mean of primer coverage for each order (r=0.09, p=0.62).

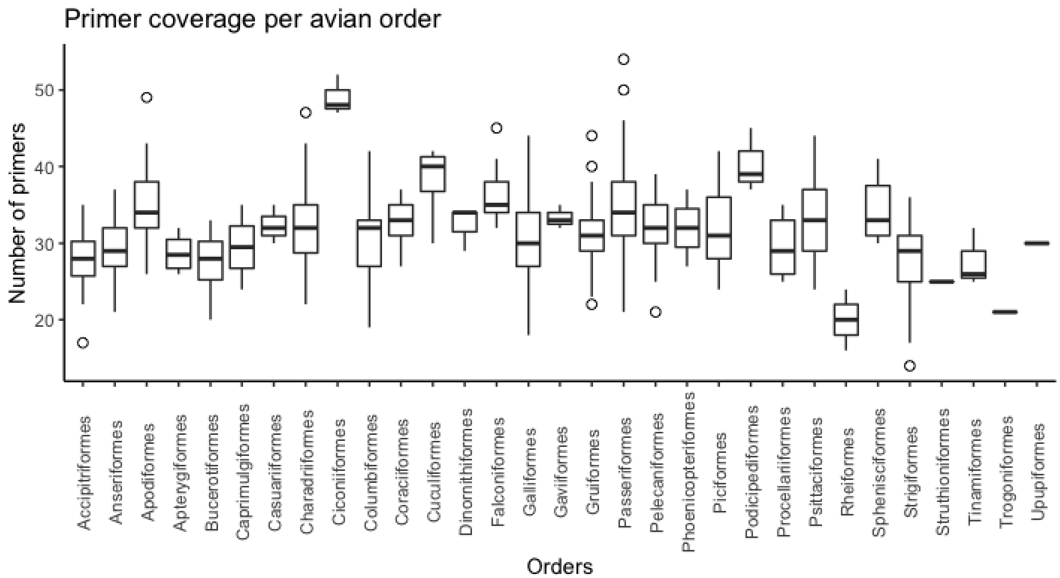

Fig. 2 Variation on the number of primers bound to the template sequences of each bird order. The dots represent data outliers.

When focusing on the individual template sequences, the ones that had the highest number of primers bound to them were *Abrornis proregulus* (Passeriformes, n=54, 45.76%), *Ciconia nigra* (Ciconiiformes, n=52, 44.06%), *Dives dives* (Passeriformes, n=50, 42.37%), *Glaucis hirsutus* (Apodiformes, n=49, 41.52%) and *Ciconia boyciana* (Ciconiiformes, n=48, 40.67%). The ones that had the fewest number of primers bound to them were *Glaucidium brodiei brodiei* (Strigiformes, n=14, 11.86%), *Rhea americana* (Rheiformes, n=16, 13.55%), *Butastur liventer* (Accipitriformes) and *Ninox strenua* (Strigiformes) (n=17, 14.40% each) and *Meleagris gallopavo* (Galliformes, n=18, 15.25%).

Allowed mismatch effect

When considering the number of allowed mismatches, the primer that could bind to the greatest number of template sequences when no mismatch was allowed was the reverse primer H8121 (n=505, 58.17%). However, when one till seven mismatches were allowed, the most sequence-bound primers were AvMiF1 (n=336, 38.70%), mCOIR2 (n=342, 39.40%), COIbR (n=252 and n=200, 29.03 and 23.04%), CO1F (n=205 and n=53, 23.61 and 6.10%) and ZBJ-ArtF1cfw (n=8, 0.92%), respectively. When two mismatches were allowed, we observed the highest number of primers with at least one binding event (n=104, 66.66%). When up to seven mismatches were allowed, the lowest number of primers with at least one binding event could be detected (n=6, 3.84%) (Figure 3). It was possible to notice that the number of primers bound to at least one template sequence increased with the increasing number of primer-template mismatches up to two mismatches allowed (Figure 3). After that, with three or more mismatches allowed, the opposite occurred, where fewer primers could bind to the template sequences. There was a significant negative correlation between the number of mismatches allowed and the number of primers with at least one binding event, as shown in Figure 3 ($r = -0.87$, $p < 0.01$), meaning that, as the number of allowed mismatches increased, the number of primers with at least one binding event decreased.

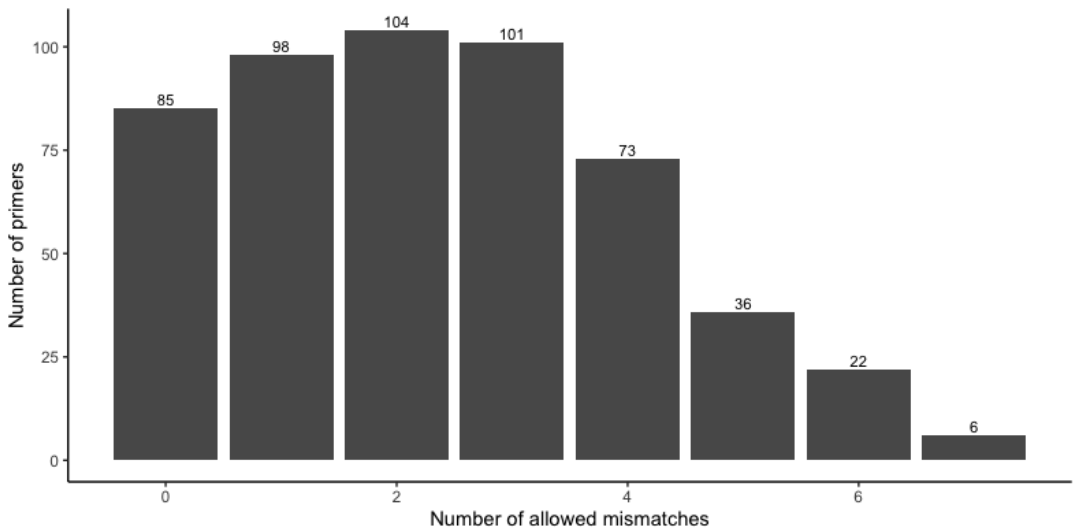

Fig. 3 Effect of the number of allowed primer-template mismatches on primer binding. Y-axis = Number of primers with at least one binding event on every scenario of allowed mismatches (X-axis).

Binding position

Most of the primers were bound to the first 800bp of the COI gene (Figure 4). Most forward primers were focused on the first 300bp of the gene and of the reverse primers after 300bp (Figure 4). Only four primers were bound after the 1000bp of this gene (L7945, L6615(tTyr)_COI, H8121, BirdR3 - Figure 4).

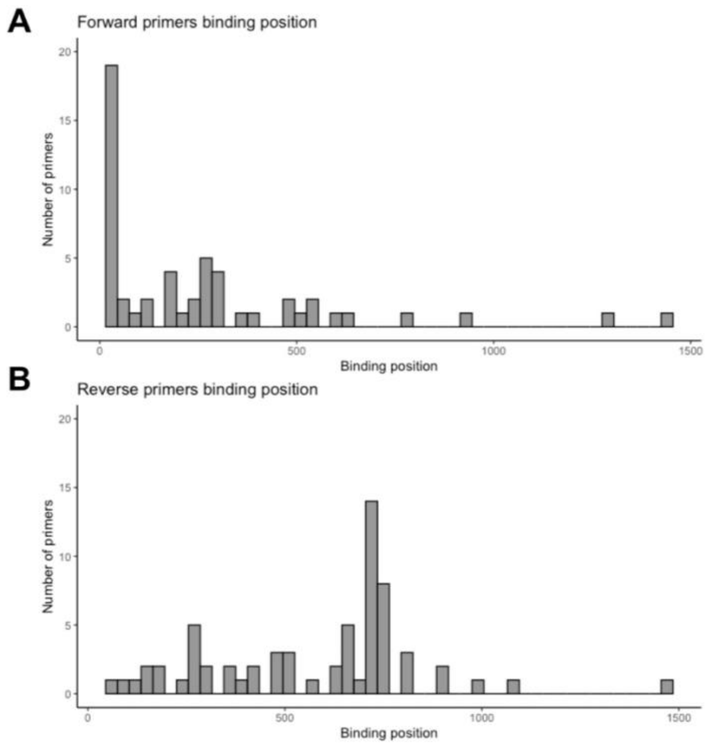

Fig. 4 Primers binding position distributed along the 1,500pb of the avian COI gene. A) Binding position of forward primers. B) Binding position of reverse primers.

PCR physicochemical properties

The average melting temperature for the analyzed primers was 55.54ºC. Constraints regarding primers GC content, overall content (GC ratio), and number of GCs on the 3' end (GC clamp) had an average of 0.47 and 1.12, respectively (Supplementary Figure 2). The average number of homopolymers runs (mononucleotide repeats – Number of runs) and dinucleotide repeats (Number of repeats) were 3.24 and 0.81, respectively. Properties regarding secondary structures, self, and cross dimerization, had a mean value of free energy (ΔG) of -0.35, -1.91, and -6.27, respectively (Supplementary Figure 2).

Primer filtering phase

In the filtering phase, primers were checked either to pass or not on the range and limits of the analyzed constraints (Supplementary Table 2). Out of the 156 analyzed primers, 118 primers passed the coverage constraint for presenting at least one binding event, 151 showed the desired primer length, and 90 were set within the range for the GC clamp and 122 for the GC ratio. On the stability constraints, 137 passed the number of runs, and all of them passed the number of repeats. The desired value of free energy to prevent secondary structures, self-dimerization, and cross dimerization comprised 130, 147, and 50 primers. Lastly, 138 primers were within the desired melting temperature range. When checking all constraints together, only 19 primers passed all of them, and therefore, were within the all limits and settings range established by this analysis (Table 2).

Table 2. Name, orientation, original reference of the 19 primers that fulfilled all the analyzed constraints range, and limits. N: Number of template sequences covered by the primer.

Primer name	Orientation	N	Reference
AWCintR6	Reverse	44	Patel et al. 2010
AWCintF5	Forward	5	Patel et al. 2010
BirdR2	Reverse	10	Hebert et al. 2004
H7390Thrush	Reverse	401	Saitoh et al. 2015
Pel-F2-COI	Forward	1	Nikulina & Schmolcke 2015
Pel-R2-COI	Reverse	167	Nikulina & Schmolcke 2015
BirdR1-Ixmod	Reverse	549	Paeckert et al. 2014
BirdR3	Reverse	62	Hebert et al. 2004
AF	Forward	88	Park et al. 2011
BirdF4	Forward	88	Hebert et al. 2004
PasserF1	Forward	59	Lohman et al. 2009
COIbF	Forward	2	Bitaniyi et al. 2011
BC_65	Forward	388	Ogawa et al. 2015
FF2	Forward	40	Ivanova et al. 2007
COI-L1	Forward	145	Fleischer et al. 2006
VF1	Forward	351	Ward et al., 2005
VF1d	Forward	393	Ivanova et al., 2006
VF1_t1	Forward	65	Ivanova et al., 2006
VF1d_t1	Forward	393	Ivanova et al., 2006

Primers subset

When gathering the top 10 forward and reverse primers regarding template sequence coverage and the 19 primers that could fulfill all the constraints range on the filtering phase, five primers subsets were suggested to maximize template coverage (Table 3). No subset alone could cover all template sequences, with the percentage of sequences covered by these primers subsets ranging from 1.15% to 90.32% (Table 3). However, when combining two primer subsets, the complete coverage of avian COI sequences here analyzed was achieved (Figure 5). Any of the three primer subsets (BC_65 + H8121, BHRM + mCOIR2, and BC_65 + AvesR) with similar coverage percentages (Table 3) can be combined with the subset with the highest coverage percentage (FF2d + CO1R) to accomplish all template sequences coverage (Figure 5). Figure 5 shows us that with only two primer subsets we can achieve the coverage of all avian species with complete mitochondrial genomes available and using more than two sets, the results can be redundant.

Table 3. Primer subsets suggested among the most coverage breadth and the fulfilled constraints for maximizing template sequence coverage. N is the number of template sequences covered by the primer set and its respective percentage of covered template sequences.

Primer subset	Forward primer sequence	Reverse primer sequence	N	%
BirdHRM-F + mCOIR2	CACGAATAAACAACATAAGCTTCTG	CAGGGTGTCCGAAGAATC	632	72.81%
FF2d + CO1R	TTCTCCACCAACCACAARGAYATYGG	ACTTCTGGGTGGCCAAAGAATCAGAA	784	90.32%
BirdF1d + BirdR1dt	TCAACCAACCACAAAGAYATYGGYAC	ACGTGGGAGATGATTCCGAAKCCKGG	10	1.15%
BC_65F + H8121	TTCTCAACCAACCACAAAGAYATYGG	GGGCAGCCRTGRATTCAYTC	623	71.77%
BC_65F + AvesR	TTCTCAACCAACCACAAAGAYATYGG	AAGATGTAGACTTCTGGGTG	544	62.67%

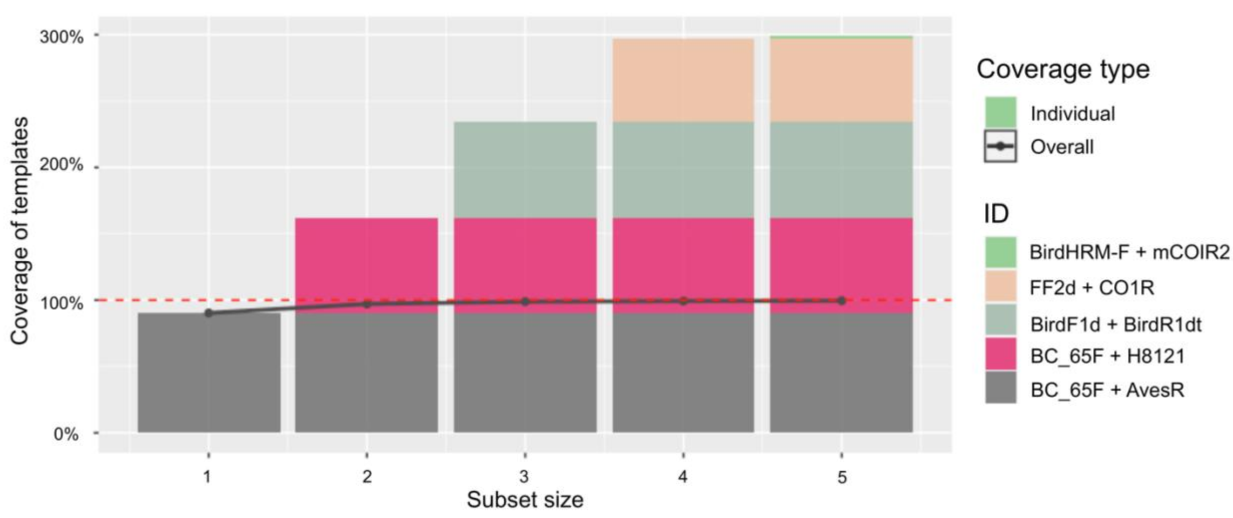

Fig. 5 Percentage of template sequences coverage of the primer subsets analyzed on the second evaluation round. The red dashed line represents the complete coverage of the analyzed template sequences (100%). Two subsets of primers were capable of covering all avian COI sequences analyzed in silico here, with the use of three or more primer sets being redundant in terms of template sequence coverage.

4. Discussion

Data retrieval

From the papers retrieved, we observed that the COI gene had been widely used on the molecular identification of birds, with studies varying from solving unresolved taxonomic phylogenetic relationships (Tritsch et al. 2017) to the identification of roadkill carcass (Rodríguez-Castro et al. 2017) and fertile hybrid in a wild population (Kleven et al. 2020). The primers most frequently used for this purpose are the ones designed by the research group that named and popularized the technique of molecular identification of species as DNA barcoding, probably because they were one of the first primers designed to amplify and access the COI gene diversity in a wide sample of bird orders after the term DNA barcode was coined (Hebert et al. 2004). The older published primers are potentially the most frequently used when retrieving existing primer to be used in other studies, as shown here.

Regarding the number of mitochondrial genome sequences available for the avian orders, of the 11,158 recognized species of birds (BirdLife-International 2019), we could observe only 7.78% (n=868) of its species with sequences available, showing that there's still a lot of genomic information to be generated for the group of birds. Passeriformes had more than 13 times the average number of template sequences available for all the bird orders (mean=28) (Figure 1B). This can be explained by the fact that this order has the highest species richness of the group, and solely, it represents more than 59% of the avian species (BirdLife-International 2019). The passerines group has shown to be outnumbered on sampling not only in genomic databases but also in museum collections (Billerman and Walsh 2019) which are also important sources for retrieving historical genetic data. However, because of this large number of species, Passeriformes still has a considerable lack of genomic information since out of its 6,633 recognized species (BirdLife-International 2019), only 5.63% of it have mitochondrial genome sequences available (n=374).

Other bird orders with lower number of species when compared to Passeriformes had greater percentage of its total species with mitochondrial genome sequences available, such as Galliformes (27.92%) and Psittaciformes (14.52%). Galliformes, the second order with the highest number of available mitochondrial sequences (n=86) is one of the orders most related to humans, with widely domesticated species with great economic importance (Peters 2014) and hunting target species for food, trading, and plumage (Fernandes-Ferreira et al. 2012; Fuller and Garson 2000). Galliformes also comprises the chicken species which are widely used as model organisms for many genetics, immunology and developmental studies (Serebrovsky and Petrov 1930; Stern 2005) and was also the first avian species to have its complete genome sequenced in 2004 (Hillier et al. 2004).

Psittaciformes, with 61 complete COI sequences publicly available, is the avian order with most reports within the international illegal pet trade (Bush et al. 2014) and therefore, demands many studies regarding molecular identification to support wildlife traffic surveillance (Arenas et al. 2017; Gonçalves et al. 2015). With worldwide distribution, extensive migration strategy, and diverse breeding system, Charadriiformes is the fourth order with the greatest number of mitochondrial genome sequences available due to the vast number of studies on migration, speciation, and ecology supported by the molecular analysis performed for this group (Baker et al. 2007).

On the other hand, there is a lack of genomic information for some bird orders. Cariamiformes, Coliiformes, Galbuliformes, Musophagiformes, and Opisthocomiformes did not have their complete or partial mitochondrial genome available, and Rheiformes had the lowest number of primers bound to its template sequences. These six orders with gaps of knowledge have specimens located restrictedly in Neotropics and sub-Saharan Africa, which are regions known either for the low research investment or for the great number of civil armed conflicts (Ansorg 2014) which, contribute to underdevelopment regions (Augustine 2018) and consequently, to fewer studies for their own species (Ducatez and Lefebvre 2014). Also, the small number of species represented by these orders, as well as their small geographic distribution (BirdLife-International 2019) go in agreement with the fact that orders with low diversity of species and species with narrow habitat breadth have lower research effort (Ducatez and Lefebvre 2014).

In silico primer evaluation

A prior in silico analysis of a desired primer set can be essential to orientate and personalize a study when evaluating the best sets that cover most of the taxonomic group of interest and show the best physicochemical properties for increasing the chances of laboratory amplification success. The average primer length was 22.89bp and primer length was filtered up to 30bp because longer primers are recommended for amplifying regions with a high degree of heterogeneity (Dieffenbach et al. 1993). However, primers with exceedingly long sequences can also be more prone to forming primer-dimers and should be avoided (Rychlik 1993).

On a previous round of analysis, allowing primer-template mismatch on the 3’ end did not significantly alter the number of primers with at least one binding event (data not shown), so we chose to keep for the following analysis, mismatches on the last seven bases of the primer’s 3’ end forbidden because primer-template mismatches on this region have already been proved to drastically reduce primer efficiency and consequently, PCR amplification product (Kwok et al. 1990; Stadhouders et al. 2010; Whiley and Sloots 2005). A significant negative mismatch effect was observed when the number of allowed mismatches increased, and the number of primers bound to at least one template sequence decreased (Figure 3). The presence of multiple primer-template mismatches can drastically reduce the polymerase efficiency of effectively extending over a mismatched primer-template duplex and increase the chances of occurring consecutive mismatches, which can lead to very inefficient amplification or no amplification at all (Kwok et al. 1990; Stadhouders et al. 2010), a situation that was also clearly observed here on this in silico primer and PCR evaluation.

None of the five primers that could cover the highest number of template sequences passed on the filtering phase (Table 1 & 2). The free energy available for cross dimerization was the constraint that most of these top 5 primers could not fulfill (mCOIR2, K_Bird_F1, VR1, BirdF1d), followed by GC clamp (AWCintR4, VertebrateR1 & VR1) and free energy available for forming primer secondary structures (VertebrateR1). Cross dimerization was also the constraint in which fewer primers fit the desired range. It needs to be avoided because primer-dimers like this can cause PCR yield to be reduced or to be resulted from primers self-amplification (Dieffenbach et al. 1993). However, just because the most coverage broad primers did not fulfill all the analyzed constraints, that does not mean they will not yield specific PCR products. This can be overcome by adjusting some PCR conditions, such as temperature and duration of PCR cycles (Rychlik 1993).

With this analysis, we could also see that the first three primers that bound to the highest number of template sequences were not the most order coverage broad because they could not cover all template sequences of all avian orders (Table 1). However, the orders not covered might have a limitation on their studied sequence diversity due to the low number of available sequences (STable 4 & Figure 1B). In addition, the seven primers that bound to template sequences of only one avian order might have been influenced by the low number of genomic sequences available for these orders, which might have presented different results with other set of data. Some initiatives that try to overcome the lack of sequences available for these groups, such as the Earth Biogenome Project, that aims to sequence, catalog and characterize the genomes of all Earth's eukaryotic biodiversity over a period of ten years (Lewin et al. 2020), should help studying groups with high sequence diversity that requires greater number of available sequences.

The average values of all physicochemical constraints were within the desired range established for this analysis and are important constraints that should be previously evaluated when choosing an existing primer set or designing new ones because fulfilling these constraints range values can increase the chances of yielding great PCR results (Kumar and Chordia 2015). The free energy for cross dimerization was the constraint that most primers could not fulfill. However, because the calculation of this constraint depends on the interaction between primers, its final value can vary according to the primers included in the evaluation analysis (Kreer et al. 2020). For this reason, evaluated primers should be analyzed within larger and smaller sets of the desired primers because, as individual sets, the primers can perform differently than when evaluated with a larger array.

Only 19 primers could fit all the physicochemical properties range at the same time. However, of these primers, only six could bind to more than 40% of the template sequences, with BirdR1-lxmod being the most coverage broad among them (n=549, 63.04%) (Table 2). Of these six primers, five of them showed some level of degeneracy in its sequence, which might be why they were the most coverage broad among the filtered primers. Since no primers could cover all template sequences and fulfill all physicochemical properties at the same time, we performed a second round of evaluation with the primers that either covered the highest number of template sequences or fit all the evaluated constraints range on the first evaluation. With the second analysis, we could suggest primer subsets that, when used together, should retrieve the best coverage and PCR performance for the avian COI gene for 7.78% of all avian species of the world (Table 3). This subset selection shows that for covering all avian COI sequences available, no more than two primer sets are needed (Figure 5) since the complete coverage is achieved with this number and using more than that is not necessary and can be time and money consuming.

Most of the primers covered the first 800 bp of the COI gene, which comprehends the well-known and accepted region used for molecular identification, known as the barcode region, which has enough nucleotide differences to be used for this purpose (Hebert, Cywinska, et al. 2003). Although this barcode region of the COI gene is widely used on the molecular identification of birds (Gonçalves et al. 2015; Hebert et al. 2004; Tavares et al. 2011) it has shown higher intraspecific diversity than interspecific diversity in groups with recent divergence, like the genus *Sporophila* within Thraupidae (Campagna et al. 2010). Therefore, for these cases, other barcode regions of different genes should be adopted. The barcode region amplified by the primers analyzed here, especially the optimal subsets, can also be further evaluated for taxonomic resolution within different levels of molecular identification (Bylemans et al. 2018).

5. Conclusion

The COI gene is being widely used on the molecular identification of birds for different purposes ever since its recommendation of universal animal barcode region in 2004 by Paul Hebert and his team. Thus, many primers are available for accessing this region, not all of them seem to be solely capable of fulfilling complete coverage and the PCR physicochemical properties desired range. Therefore, it is crucial to evaluate the primers in silico performance and taxonomic coverage before testing in laboratory experiments to maximize the coverage range, guide personalized analysis, and avoid PCR issues. With the approach performed on this work, we were able to visualize the individual and joint coverage range as well as the physicochemical properties and PCR performance of the current primers designed for accessing the avian COI gene. Also, we were able to find optimal primer subsets for more specific or broad objectives in order to guide researchers in the process of choosing the best primer set for each individual goal.

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Table 1. Top 5 primers (overall, forward, and reverse) with the highest number of covered template sequences. Every template sequence covered by a primer is considered a primer-template binding event. NO and N refer to the number

of avian orders and the number of template sequences covered by the primer, respectively. %: percentage of template sequences covered by the primer.

Table 1. Name, orientation, original reference of the 19 primers that fulfilled all the analyzed constraints range, and limits. N: Number of template sequences covered by the primer..

Table 2. Primer subsets suggested among the most coverage breadth and the fulfilled constraints for maximizing template sequence coverage. N is the number of template sequences covered by the primer set and its respective percentage of covered template sequences.

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Fig. 4 Primers binding position distributed along the 1,500pb of the avian COI gene. A) Binding position of forward primers. B) Binding position of reverse primers.

Fig. 5 Percentage of template sequences coverage of the primer subsets analyzed on the second evaluation round. The red dashed line represents the complete coverage of the analyzed template sequences (100%). Two subsets of primers were capable of covering all avian COI sequences analyzed in silico here, with the use of three or more primer sets being redundant in terms of template sequence coverage.

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Supplementary Table 1. Reference information about COI primers retrieved from 103 papers and its proper original references. Primers that had no given name were named after its original designing author.

Supplementary Table 2. Input and coverage constraints and PCR conditions evaluated, as well as its target range and limits stablished as settings for the evaluation of primers designed to access avian COI gene.

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Supplementary Table 4. Number of template sequences covered by each primer that presented at least one binding event on this in silico COI coverage evaluation analysis. Number of template sequences covered by each primer per bird order and as a total per primer.

Supplementary Fig. 1 Usage frequency of primers for the COI gene used on birds DNA barcoding studies and its year of publication. A significant negative relation between the primers frequency of usage and its year of publication was observed ($r= -0.17$, $p = 0.01$).

Supplementary Fig. 2 Distribution of the evaluated coverage and input constraints as well as the PCR physicochemical properties range of the evaluated COI primer sets available for the avian group. A) Distribution of the primers' observed melting temperature (ºC); B) Distribution of the primers' length (in bp); C) Level of degeneracy observed on the retrieved primers; D) Distribution of the values of free energy available (ΔG) for the formation of primer secondary structures (kcal/mol); E) Distribution of the values of free energy available (ΔG) for the interaction of a primer with itself (kcal/mol); F) Distribution of the values of free energy available (ΔG) for the interaction of a primer with another primer (kcal/mol); G) Distribution of the primers' GC ratio in terms of number in the interval [0,1]; H) Distribution of the GC at the 3' end (GC clamp) observed on the evaluated primers; I) Distribution of the length of homopolymer runs in the evaluated primers (Number of runs – in bp); J) Distribution of the length of dinucleotide repeats in the evaluated primers (Number of repeats – in bp).

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Reference information about COI primers retrieved from 103 papers and its proper original references. Primers that had no given name were named after its original designing author.

Author & year	Year	Forward primers		Reverse primers		Original reference
		Name	Sequence	Name	Sequence	
Tizard et al. 2019	2019	AWCF1	CGCYTWAACAYTCYGCCATCTTACC	AWCR6	ATTCCTATGTAGCCGAATGGTTCTTT	Patel et al. 2010
		AWCF1	—	AWCR3	ATGCTCGGGTGTCTACGTCTAT	
		AWCF1	—	AWCintR2	ATGTTGTTTATGAGTGGGAATGCTATG	
		AWCintF2	ATAATCGGAGGCTTCGGAAACTGA	AWCintR4	TGGGAKAGGGCTGGTGGTTTTATGTT	
		AWCintF4	TCCTCAATCCTGGGAGCAATCAACTT	AWCintR6	GGATTAGGATGTAGACTTCTGGGTG	
		AWCF1	—	AWCintR1	CCTGGTTGACCTAGTTCTGCTCG	
		AWCintF1	CCGAGCAGAACTACGTCAACC	AWCintR2	—	
		AWCintF3	ATCGGAGCCCCAGACATAGCATT	AWCintR3	TTGATGGCTGTTGTGATAAAGTTGAT	
		AWCintF4	—	AWCintR5	TGCTGGGTCGAAGAATGTGGTGTT	
		AWCintF5	GGCATCACCATACTACTAACAGACCG	AWCintR6	—	
González-Varo et al. 2019	2019	COI-fsdF	GCATGAGCCGGAATAGTRGG	COI-fsdR	TGTGAKAGGGCAGGTGGTTT	González-Varo et al. 2014
		COI-fsdF	—	COI-fsd-degR	GTTGTTTATTCGGGGGAATG	
		COI-fsd-degF	GGAGCCCCAGACATAGCAT	COI-fsdR	—	
Cheon et al. 2018	2018	BirdF1	TTCTCCAACCACAAAGACATTGGCAC	BirdR1	ACGTGGGAGATAATTCCAAATCCTG	Hebert et al. 2004
		BirdF1	—	BirdR2	ACTACATGTGAGATGATTCCGAATCCAG	
Parejo-Farnés et al. 2018	2018	BirdF1	—	COIbirdR2	ACGTGGGAGATAATTCCAAATCCTGG	Hebert et al. 2004, Kerr et al. 2009

		BirdF1	—	AvMiR1	ACTGAAGCTCCGGCATGGGC	
		K_Bird_F1	CCCCAGACATAGCATTYCC	K_Bird_R1	TTGTGATAGTGGTGGGGTTTTAT	Joo & Park 2012
Summa et al. 2018	2018	BirdF1	—	BirdR2	—	Hebert et al. 2004
Dimitriou et al. 2017	2017	L6615(tTyr)_COI	CCYCTGTAAAAAGGWCTACAGCC	H7548 (COI)	GTDGCNGANGTRAARTADGCTCG	Sorenson et al. 1999, Dove et al. 2008
		LCO1490	GGTCAACAAATCATAAAGATATTGG	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994
		BirdF1	—	COIbirdR2	—	Hebert et al. 2004, Kerr et al. 2009
Rodríguez-Castro et al. 2017	2017	LCO1490	—	HCO2198	—	Folmer et al. 1994
Jing Li et al 2017	2017	BirdF1	—	BirdR1	—	Hebert et al. 2004
		FalcoFA	TCAACAAACCACAAAGACATCGGCAC	BirdR2	—	Kerr et al. 2007, Hebert et al. 2004
		VF1	TTCTCAACCAACCACAAAGACATTGG	VR1	TAGACTTCTGGGTGGCCAAAGAATCA	Ward et al., 2005
		VF1d	TTCTCAACCAACCACAARGAYATYGG	VR1d	TAGACTTCTGGGTGGCCRAARAAYCA	Ivanova et al., 2006
		GF_1	TCAACYAACCACAAAGATATCGGAAC	GR_1	ACGTGTGAGATGATTCCAAAACCTG	Jing Li et al., 2017
Tritsch et al. 2017	2017	BirdF1	—	BirdR1	—	Hebert et al. 2004
Gandolfi et al. 2017	2017	BirdF1	—	BirdR1	—	Hebert et al. 2004
		5-NEST-F	GTAATCGTTACAGCCCATGC	3-NEST-R	GGGTCGAAAAATGTGGTGTT	Gandolfi et al. 2017
Bilgin et al. 2016	2016	BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdF1	—	BirdR2	—	

Armani et al. 2016	2016	FISH-BCL_F	TCAACYAATCAYAAAGATATYGGCAC	FISH-BCH_R	ACTTCYGGGTGRCCRAARAATCA	Baldwin et al. 2009, Handy et al. 2011
Sugita et al. 2016	2016	L6697Bird_F	TCAACYAACCACAAAGAYATCGGYAC	H7390Thrush_R	ACGTGGGARATRATTCCAAATCCTG	Saitoh et al., 2015
Khanam et al. 2016	2016	BirdF1	—	AWCintR2	—	Hebert et al. 2004, Patel et al. 2010
Nikulina & Schmolcke, 2015	2015	Pel-F1-(COI)	GCCGTTCTACTACTACTGTCC	Pel-R1-(COI)	AGAATGTAGTGTTTAGGTTTCGGTC	Nikulina & Schmolcke, 2015
		Pel-F2-(COI)	ACTGTCCCTCCCAGTCTTAGCC	Pel-R2-(COI)	AGCAGGGTCGAAGAATGTAGTG	
		Pel-F3-(COI)	GGAGGCTTTGGAAACTGACTAG	Pel-R3-(COI)	ATACGTGGGAATGCTATGTCTGG	
Chaves et al. 2015	2015	L6615	CCYCTGTAAAAAGGWCTACAGCC	H8121	GGGCAGCCRTGRATTCAYTC	Sorenson 2003
		socoiF1	TTCTACAAACCATAAAGATATTGGCA	H6035	CCTCCTGCAGGGTCAAAGAATGT	Chaves et al. 2008
		LCO1490	—	HCO2198	—	Folmer et al. 1994
		VF1_t1	TTCTCAACCAACCACAAAGACATTGG	VR1_t1	TAGACTTCTGGGTGGCCAAAGAATCA	Ivanova et al. 2006
		VF1d_t1	TTCTCAACCAACCACAARGAYATYGG	VR1d_t1	TAGACTTCTGGGTGGCCRAARAAYCA	
		VF1i_t1	TTCTCAACCAACCAIAAIGAIATIGG	VR1i_t1	TAGACTTCTGGGTGICCIAAIAAICA	
Saitoh et al. 2015	2015	Bird F1	—	Bird R1	—	Hebert et al. 2004
		Bird F1	—	Bird R2	—	
		L6697Bird_F	—	H7390Thrush_R	—	Saitoh et al. 2015
		FalcoFA	—	VertebrateR1	TAGACTTCTGGGTGGCCAAAGAATCA	Kerr et al. 2007
González-Varo et al. 2014	2014	COI-fsdF	—	COI-fsdR	—	González-Varo et al. 2014
	2014	BirdF1-Ixmod	GGAACCGCCCTAAGCCTAC	BirdR1-Ixmod	AGGATRTAGACTTCTGGGTG	

Paeckert et al. 2014		BirdF1-Ixmod	—	BirdR2_Ixmod	CGTGGGAATGCTATGTCGG	Paeckert et al. 2014
		BirdF2_Ixmod	TYGGAGGATTYGGAAAC	BirdR3_Ixmod	ATGAAGTTGATTGCCCC	
Huynen & Lambert 2014	2014	mCOIF1b	ACAGCCCTCAGCCTACTCAT	mCOIF3	CCGATATAGCATTTCCAC	Lambert et al. 2005
		mCOIF4b	TCCATCCTAGGAGCTATCAA	mCOIR2	CAGGGTGTCCGAAGAATC	
		mCOIR3b	GGTAGGAGTCAGAAGCTTAT	mCCIR4	ATGTTAATTGCTGTGGT	
Khan e Arif 2013	2013	Bird F1	—	Bird R1	—	Hebert et al. 2004
Awan et al. 2013	2013	Bird F1	—	Bird R1	—	Hebert et al. 2004
Nijman & Aliabadian 2013	2013	BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdF1	—	BirdR2	—	
Aliabadian et al. 2013	2013	BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdF1	—	BirdR2	—	
		CO1-ExtF	ACGCTTTAACACTCAGCCATCTTACC	CO1-ExtR	AACCAGCATATGAGGGTTCGATTCCT	Johnsen et al. 2010
Breman et al. 2013	2013	Bird1Fd	TCAACCAACCACAAAGAYATYGGYAC	BirdH_351d_370d	CCTGCTCCWGCT TCTAYDGT	Breman et al., 2013, Sonet et al. 2011
		Aves_L288_310	CGCATAAACAACATAAGCTTCTG	BirdR1dt	ACGTGGGAGATGATTCCGAAKCCKGG	Louette et al. 2011, Breman et al., 2013
Hogner et al. 2012	2012	Aldob-6	AGACCATGATCTCCAGCGCT		CCTTCCAGGTAGACATGATG	Bourge et al. 2005
		Brm-15	AGCACCTTTGAACAGTGGTT		TACTTTATGGAGACGACGGA	
		COI-ExtF	—	BirdR2	—	Johnsen et al. 2010, Hebert et al. 2004
		PhSa-F1	AACGTAGTCGTCACAGCCCATGCTT	PhSa-R1	TTATTCGRGGRAATGCTATG	Hogner et al. 2012
		L437	CTCACGAGAACCGAGCTACT	H1248	CATCTTCAGTGTCATGCT	Tarr 1995
Abe et al. 2012	2012	BirdF1	—	BirdR1	—	Hebert et al. 2004
Tavares et al. 2011	2011	LTyr	TGTAAAAAGGWCTACAGCCTAACGC	COI907aH2	GTRGCNGAYGTRAARTATGCTCG	Tavares & Baker 2008
		LTyr	—	COI748Ht	TGGGARATAATTCCRAAGCCTGG	

Walsh et al. 2011	2011	BirdF1	—	BirdR2	—	Hebert et al. 2004
Park et al. 2011	2011	BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdF1	—	BirdR2	—	
		BirdF1	—	BirdR3	GGAGTTTGCTAGTACGATGCC	
		AF	AACCAACCACAAAGACATTGG	AR	CCATGTAGCCGAATGGTTCT	Park et al. 2011
Ong et al. 2011	2011	Bird F1	—	Bird R1	—	Hebert et al. 2004
Arif et al. 2011	2011	Uni-MinibarF1	TCCACTAATCACAARGATATTGGTAC	Uni-MinibarR1	GAAAATCATAATGAAGGCATGAGC	Meusnier et al. 2008
Arif et al. 2011	2011	Bird F1	—	Bird R1	—	Hebert et al. 2004
Yu & Chen et al. 2011	2011	P1	CCTCTGTAAAAAGGACTACAGCC	Downstream	GTTCGATTCCTTCCTTTCTTGT	Yu & Chen et al. 2011
Sonet et al. 2011	2011	BirdF1d	—	BirdR1d	—	Sonet et al., 2011, Lohman et al. 2008
		BirdF1d	—	Bird- H351d	CCTGCTCCWGCTTCTAYDGT	
		BirdF1d	—	BirdH153d	ACGATTACRTTGTARATYTGRTC	
Waugh et al. 2011	2011	AWCF1	—	AWCR4	—	Patel et al. 2010
		AWCF1	—	AWCR6	—	
Kerr 2010	2010	PsEmpCOIF	ACCTGGGCCGGTATGATT	PsEmpCOIR	GATGGCCGAAGAATCAGAAA	Kerr 2010
Chung et al. 2010	2010	BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdF1	—	BirdR2	—	
		BirdF4	AACCAACCACAAAGACATTGG	BirdR4	CCATGTAGCCGAATGGTTCT	
Johnsen et al. 2010	2010	BirdF1	—	BirdR1	—	Hebert et al. 2004, Kerr et al. 2009, Johensen et al. 2010
		BirdF1	—	BirdR2	—	
		BirdF1	—	COIbirdR2	—	
		CO1-ExtF	—	CO1-ExtR	—	
Cai et al. 2010	2010	Bird F1	—	Bird R1	—	Hebert et al. 2004, Kerr et al. 2007
		Bird F1	—	Bird R2	—	
		FalcoFA	—	Bird R1	—	

Patel et al. 2010	2010	AWCF1	—	AWCR6	—	Patel et al. 2010
		AWCF1	—	AWCR3	—	
		AWCF1	—	AWCintR2	—	
		AWCintF2	—	AWCintR4	—	
		AWCintF4	—	AWCintR6	—	
		AWCF1	—	AWCintR1	—	
		AWCintF1	—	AWCintR2	—	
		AWCintF3	—	AWCintR3	—	
		AWCintF4	—	AWCintR5	—	
		AWCintF5	—	AWCintR6	—	
Campagna et al. 2010	2010	BirdF1	—	COIbirdR2	—	Hebert et al. 2004, Kerr et al. 2009
		AvMiF1	CCCCCGACATAGCATTCC	AvMiR1	ACTGAAGCTCCGGCATGGGC	
Khan et al. 2010	2010	Bird F1	—	Bird R1	—	Hebert et al. 2004
Kerr et al. 2009	2009	BirdF1	—	COIbirdR2	—	Kerr et al. 2009
		AvMiF1	—	AvMiR1	—	
Seabrook-Davison et al. 2009	2009	cCOIF1ii	AAGGACTACAGCCTAAC	cCOIR1ii	ACGAGTCAATTTCCGAAG	Seabrook-Davison et al. 2009
		cCOIF2ii	GTAATYGTCACAGCCCATG	cCOIR2ii	GAAAAGATGGCTAGRTCTAC	
		cCOIF3iii	TTAGCYGGYAACCTAGCCCA	cCOIR3iii	AGGGTCGAAGAATGTGGTGTT	
Efe et al. 2009	2009	LCO1490	—	HCO2198	—	Folmer et al. 1994
Sheldon et al. 2009	2009	PasserF1	CCAACCACAAAGACATCGGAACC	PasserR1	GTAAACTTCTGGGTGACCAAAGAATC	Lohman et al. 2009
Aliabadian et al. 2009	2009	Bird F1	—	Bird R1	—	Hebert et al. 2004
		Bird F1	—	Bird R2	—	
Lohman et al. 2009	2009	Bird F1	—	Bird R1	—	Hebert et al. 2004
		PasserF1	—	PasserR1	—	

Dove et al. 2008	2008	CO1F	TTCTCGAACCAGAAAGACATTGGCAC	CO1R	ACTTCTGGGTGGCCAAAGAATCAGA A	Dove et al. 2008
Tavares & Baker 2008	2008	LTyr	—	COI907aH2	—	Tavares & Baker 2008
		COIaRt	ACAAACCACAAAGATATCGG	COI748Ht	TGGGARATAATTCCRAAGCCTGG	
		LTyr	—	COI745h2	ACRTGNGAGATRATTCCRAANCCNG	
Rudnick et al. 2007	2007	Bird F1	—	EagleR2b	ATTGATRGCGGTTGTGATAAA	Hebert et al. 2004 and Rudnick et al. 2007
		Bird F1	—	Bird R1	—	
Kerr et al. 2007	2007	Bird F1	—	Bird R1	—	Hebert et al. 2004 and Kerr et al. 2007
		Bird F1	—	VertebrateR1	—	
		FalcoFA	—	Bird R1	—	
Lambert et al. 2005	2005	mCOIF1b	—	mCOIF3	—	Lambert et al. 2005
		mCOIF4b	—	mCOIR2	—	
		mCOIR3b	—	mCCIR4	—	
Fieldman & Omland 2005	2005	LCO1490	—	HCO2198	—	Folmer et al. 1994
Huang, Z. & Ruan, R. 2018	2018	AvTrpF1	GGCCTTCAAAGCCTTAAAYAAGAGTT	AvSerR1	RRGGWWCGAYTCCTTCCTTTCTT	Thiemann et al. 2012
Huang et al. 2015	2015	L6772_F	TTAGCCTCCTCATTCGAGCAGAATTGGG	H7539_R	CATCTGTGGGCTCGGATGAAATGTAG	Webb & Moore 2002
		L6958_F	AATAACATAAGCTTCTGACT	H8191_R	CCAICITIIGAGGGTTCGATTCCTTCC	
		L7165_F	ACCGCCATCAACATAAAACCCCC		—	
		L7444_F	TACTCCGGAAAAAAAGAACC		—	
		L7551_F	CCGTAGGAATGGACGTTGACACCCGAGC		—	
		L7945_F	CCCCAACACTTCCTCTGCCTAGC		—	
Huang & Ke 2015	2015	L6615	—	H7032_R	TTGCCAGCTAGTGGGGGT	Sorenson & Quinn 1998

Malvika et al. 2019	2019	COXF	ACTTCTGGGTGGCCAAAGAATCAGAA	COXR	TTCTCGAACCAGAAAGACATTGGCAC	Malvika et al. 2019
Dalton et al. 2019	2019	BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdF1	—	BirdR2	—	
		COIbF	TTTCAACCAACCACAAAGACATCGG	COIbR	TATACTTCAGGGTGTCCAAAGAATCA	Bitaniyi et al. 2011
Dao Dinh et al. 2019	2019	BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdF1	—	BirdR2	—	
Acosta-Rojas et al. 2019	2019	COI-fsdF	—	COI-fsdR	—	González-Varo et al. 2014
Nota et al., 2019	2019	PGMCOIFor	GCHTCHTCHAYVGTDGAAGC	PGMCOIRev	ADGADAYDCCDGCTARRTG	Nota et al., 2019
Schütz, Tollrian & Schweinsberg, 2020	2020	Schutz01F	CTATTCGTTTGATCAGTCCTAATCACTG	Schutz01R	AAAATAGATGTTGGTATAAGACAGG GTC	Schütz, Tollrian & Schweinsberg, 2020
		Schutz02F	CCCGTATAAATAACATAAGCTTT	Schutz02R	AGAAGAGACACCTGCCAAATGGAGA	
		Schutz03F	AGGGACCCTACTAGGAGATGACCAAATC	Schutz03R	TAGGAATGATGGTGGTAATAGTCAA AAG	
Kleven et al., 2020	2020	BirdF1	—	BirdR2	—	Hebert et al. 2004
Musa et al., 2020	2020	VF1d_t1	—	VR1d_t1	—	Ivanova et al., 2006
Tsunamoto et al., 2020	2020	BirdF1	—	BirdR2	—	Hebert et al. 2004
Gaber et al., 2020	2020	aCO1-1L	AACCACAAAGAYATYGGCA	aCO1-H	GGNGGGTCTCATTTRAT	Amer, Ahmed & Shobrak 2013
Heim et al., 2019	2019	COIPreyFW	CGAGCAGARCTAGGCCAACC	COIpreyRW	GCAGGCGGTTTTATGTTGATTGCTG	Pastor-Beviá et al., 2014
		ZBJ-ArtF1c	AGATATTGGAACWTTATATTTTATTTTTGG	ZBJ-ArtR2c	WACTAATCAATTWCCAAATCCTCC	Zeale et al., 2011

Rumeu et al., 2019	2019	COI-fsdF	—	COI-fsdR	—	González-Varo et al. 2014
Martínez-López et al., 2019	2019	COI-fsdF	—	COI-fsdR	—	González-Varo et al. 2014
Tseren-Ochir et al., 2018	2018	BirdF1	—	BirdR1	—	Hebert et al. 2004
		AvesF	GCATGAGCAGGAATAGTTGG	AvesR	AAGATGTAGACTTCTGGGTG	Lee et al., 2010
Pavia et al., 2018	2018	BirdF1	—	BirdR1	—	Hebert et al. 2004, Kerr et al. 2009, Johensen et al. 2010
		BirdF1	—	BirdR2	—	
		BirdF1	—	COIbirdR2	—	
		CO1-ExtF	—	CO1-ExtR	—	
González-Varo et al., 2017	2017	COI-fsdF	—	COI-fsdR	—	González-Varo et al. 2014
Onuma et al., 2017	2017	BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdHRM-F	CACGAATAAACAACATAAGCTTCTG	BirdHRM-R2	GAATGTGGTGTTTAGGTTTCGGTC	Onuma et al., 2017
Schaffer, Zachos & Koblmuller 2017	2017	St_f	CYNCWAMCCACAARGAYATNGGNAC	St_r	GAARATYATNAYGAANGCRTGNGC	Schaffer, Zachos & Koblmuller 2017
		In1f	GGNGAYGAYCARATNTACAATGT	In1r	GGNGGNAGNAGTCARAARC	
		A2f1	CCNGACATRGCNTTCCCNCG	A2r1	GCNARGTCNACNGANGCNCCNG	
		A2f2	GNGCNCCNGAYATRGCNTTYCC	A2r2	CNGCNAGRTGNAGNGARAARATNGC	
		M2f	GGNAAYTGACTNGTNCCNCT	M2r	CNGCRTGNGCNAGRTTNCC	
		In3f	GGNGTNGGNACNGGNTGAAC	In3r	GATCANACGAANAGNGGNGTYTG	
		M3f	GGNACNGGNTGAACNGTNTACC	M3r	GRTATTGNGANATNGCNGGNGG	
		A4f	CNTCNATCCTNGGNGCAATYAAC	A4r	GCNGGGTCRAAGAANGTNGTGTT	
		M4f	GCNGGNGTNTCNTCNATTYTAGG	M4r	ARGTTGTRTTYARGTTNCGGTCYGT	

		A5f	GGNATCACNATRCTNCTNACNGACCG	E_r1	GTRKGAGATRATTCCGAAKCC	
		A5f	—	E_r2	ATNCCTATGTANCCGAATGGRTCTTT	
		M5f2	CNGTNCTAGCNGCYGGNATYACNAT	E_r1	—	
		M5f2	—	E_r2	—	
		M5f1	CNCARTAYCAAACNCCNCTNTTYGT	E_r1	—	
		M5f1	—	E_r3	TANACNTCNGGNTGNCCNAANAATC A	
Mendoza et al., 2016	2016	FalcoFA	—	VertebrateR1	—	Kerr et al., 2007, Hebert et al., 2004
		FalcoFA	—	Bird R2	—	
Joo et al., 2016	2016	K_Bird_F1	—	K_Bird_R1	—	Joo & Park, 2012
		Uni-MinibarF1	—	Uni-MinibarR1	—	Meusnier et al. 2008
Morales-Contreras et al., 2016	2016	LTyr	—	H8205	GGTTCGATTCCTTCCTTTCTTG	Tavares & Baker, 2008; Pereira & Baker, 2004
Heller et al., 2016	2016	BirdF1	—	COIbirdR2	—	Kerr et al., 2009
		AvMiF1	—	AvMiR1	—	
Ogawa et al., 2015	2015	BC_65F	TTCTCAACCAACCACAAAGAYATYGG	BC_294R	GGGACTARTCAGTTYCCRAATCC	Ogawa et al., 2015
		BC_221F	GCCCAYGCCTTYGTAATAATCTTCTT	BC_479R	TAGGTCTACTGAGGCGCCAGCATGGG C	
		BC_392F	ACAGTAGAAGCYGGAGCAGGCAC	BC_639R	GTGAGAGTAGTAGTARGACGGC	
		BC_581F	CAAACCCCCCTATTCGTATGRTCCGT	BC_813R	ACGTGGGAGATGATTCCGAAKCCTG	
Amouret et al., 2015	2015	PasserF1	—	PasserR1	—	Lohman et al., 2009
Gonçalves et al., 2015	2015	LTyr	—	COIH7557	GGCGGATGTGAAGTATGCTCGGG	Tavares & Baker 2008, Hebert et al., 2004
		LTyr	—	BirdR1	—	

Colihueque et al., 2015	2015	BirdF1	—	BirdR1	—	Hebert et al. 2004
Kováts & Ács 2013	2013	BirdF1	—	COIbirdR2	—	Kerr et al., 2009
Campbell et al., 2013	2013	VF1	—	VR1	—	Ward et al., 2005
		VF1d	—	VR1d	—	Ivanova et al., 2006
		VF1i	TTCTCAACCAACCAIAAIGAIATIGG	VR1i	TAGACTTCTGGGTGICCIAAIAAICA	
		FF2d	TTCTCCACCAACCACAARGAYATYGG	FR1d	CACCTCAGGGTGTCCGAARAAYCARAA	Ivanova et al., 2007
Huang et al., 2013	2013	BirdF1	—	BirdR2	—	Hebert et al. 2004
Reisen, Lothrop & Thiemann 2013	2013	VF1_t1	—	VR1_t1	—	Ivanova et al., 2006
		VF1d_t1	—	VR1d_t1	—	
		VF1i_t1	—	VR1i_t1	—	
		VF1	—	VR1	—	Ward et al., 2005
		BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdF1	—	BirdR2	—	
Kerr & Dove 2013	2013	BirdF1	—	COIbirdR2	—	Kerr et al., 2009
		AvMiF1	—	AvMiR1	—	
		LTyr	—	COI907aH2	—	Tavares & Baker 2008
Joo & Park 2012	2012	K_Bird_F1	—	K_Bird_R1	—	Joo & Park, 2012
Sasikala et al., 2012	2012	BirdF1	—	Bird R1	—	Hebert et al. 2004
Kwon et al., 2012	2012	BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdF1	—	BirdR2	—	
Campagna et al., 2011	2011	BirdF1	—	COIbirdR2	—	Kerr et al., 2009
		AvMiF1	—	AvMiR1	—	
Arif et al. 2012	2012	BirdF1	—	BirdR1	—	Hebert et al. 2004
Lohman et al., 2010	2010	PasserF1	—	PasserR1	—	Lohman et al., 2009

Tavares, Kroon & Baker 2010	2010	Ltyr	—	COI748Ht	—	Tavares & Baker 2008
Lifjeld et al. 2010	2010	BirdF1	—	COIbirdR2	—	Hebert et al. 2004, Kerr et al. 2009
Lee et al. 2010	2010	BirdF1	—	BirdR1	—	Hebert et al. 2004
		AvesF	—	AvesR	—	Lee et al., 2010
Alcaide et al. 2009	2009	M13BC-FW	TGTAAAACGACGGCCAGT	BCV-RV1	GCYCANAYYATNCYYRTRTA	Alcaide et al. 2009
Kerr et al. 2009	2009	BirdF1	—	COIbirdR2	—	Kerr et al. 2009
		AvMiF1	—	AvMiR1	—	
Chaves et al. 2008	2008	socoiF1	—	H6035	—	Chaves et al. 2008
		LCO1490	—	HCO2198	—	Folmer et al. 1994
Fleischer et al. 2006	2006	L6615_Fleischer	CCTCTGTAAAAAGGACTACAGC	COI-H1	CTGAGGGCTGTGCCGATTAT	Fleischer et al. 2006, Webb & Moore 2002, DeFilippis & Moore 2000
		COI-L1	ATCTTTGGTGCGTGAGCTG	COI-H2	GAAGAAGATTATTACGAATGCATGG	
		L7945_F	—	COI-H3	GCGAAGGCTTCTCAAATGAT	
		COI-L2	ACAGCCGTTATCATACTCCTATTT	H8191m	TGTGAGGGTTCGATTCCTTCC	
Hebert et al. 2004	2004	BirdF1	—	BirdR1	—	Hebert et al. 2004
		BirdF1	—	BirdR2	—	
		BirdF1	—	BirdR3	—	

Supplementary Table 2. Input and coverage constraints and PCR conditions evaluated, as well as its target range and limits stablished as settings for the evaluation of primers designed to access avian COI gene.

Input constraints

Constraint	Target range	Limit range
Coverage	1 - ∞	1 - ∞
Length	18 - 30	18 - 30
GC clamp	1 - 3	0 - 4
GC ratio	0.4 - 0.6	0.3 - 0.7
Number of runs	0 - 4	0 - 6
Number of repeats	0 - 4	0 - 6
Self dimer ΔG [kcal/mol]	-5 - ∞	-7 - ∞
Cross dimer ΔG [kcal/mol]	-5 - ∞	-7 - ∞
Structure ΔG [kcal/mol]	-1 - ∞	-2 - ∞
Tm range [°C]	50 - 70	50 - 75

Coverage constraints

Constraint	Target range
3' Mismatch position	8 - ∞
Coverage Model FPR	-∞ - 0.06
Constraint	**Settings**
Allowed mismatches	7
Allowed off-target binding ratio	1
Binding region definition	within

PCR conditions

Condition	Settings
Taq polymerase	TRUE
Na+ [M]	0
Mg2+ [M]	0.0015
K+ [M]	0.05
Primer [M]	2.00E-07

Template [M]	1.28E-11
PCR Cycles	25

Supplementary Table 3. Complete COI sequences used as references to evaluate primers developed for this gene for the bird group. Sequences were retrieved from complete or partial annotated mitochondrial genomes available on NCBI database.

Order	Species	Sequence Length	GenBank Accession Number
Accipitriformes	*Accipiter gentilis*	1551	AP010797
Accipitriformes	*Accipiter nisus*	1551	KJ680300
Accipitriformes	*Accipiter trivirgatus*	1551	MK953813
Accipitriformes	*Accipiter virgatus*	1551	KJ699124
Accipitriformes	*Aegypius monachus*	1551	KF682364
Accipitriformes	*Aquila chrysaetos*	1551	KF905228
Accipitriformes	*Aquila heliaca*	1551	KU646835
Accipitriformes	*Aquila nipalensis*	1551	MK860035
Accipitriformes	*Butastur indicus*	1551	AB830616
Accipitriformes	*Butastur liventer*	1551	AB830617
Accipitriformes	*Buteo buteo*	1551	AF380305
Accipitriformes	*Buteo buteo burmanicus*	1551	KM364882
Accipitriformes	*Buteo hemilasius*	1551	KT935541
Accipitriformes	*Buteo lagopus*	1551	KP337337
Accipitriformes	*Cathartes aura*	1551	AY463690
Accipitriformes	*Circus cyaneus*	1551	KU237286
Accipitriformes	*Circus melanoleucos*	1551	KT438620
Accipitriformes	*Gyps coprotheres*	1551	MF683387
Accipitriformes	*Gyps fulvus*	1551	KX893247
Accipitriformes	*Gyps himalayensis*	1551	KY594709
Accipitriformes	*Haliaeetus albicilla*	1551	MK043028
Accipitriformes	*Hieraaetus fasciatus*	1551	KP329567
Accipitriformes	*Milvus migrans*	1551	MG930481
Accipitriformes	*Nisaetus alboniger*	1551	AP008239
Accipitriformes	*Nisaetus nipalensis*	1551	AP008238
Accipitriformes	*Pandion haliaetus*	1551	DQ780884

Accipitriformes	*Sagittarius serpentarius*	1551	KF961184
Accipitriformes	*Spilornis cheela*	1551	JN191388
Anseriformes	*Aix galericulata*	1551	KF437906
Anseriformes	*Anas acuta*	1551	KF312717
Anseriformes	*Anas clypeata*	1551	KT345702
Anseriformes	*Anas crecca*	1551	KF203133
Anseriformes	*Anas platyrhynchos*	1551	MF069250
Anseriformes	*Anas poecilorhyncha*	1551	KF156760
Anseriformes	*Anser albifrons*	1551	AF363031
Anseriformes	*Anser albifrons frontalis*	1551	MH000287
Anseriformes	*Anser anser*	1551	MK133021
Anseriformes	*Anser cygnoides*	1551	KT427463
Anseriformes	*Anser fabalis*	1551	HQ890328
Anseriformes	*Anser indicus*	1551	KM455570
Anseriformes	*Anseranas semipalmata*	1551	AY309455
Anseriformes	*Aythya americana*	1551	AF090337
Anseriformes	*Aythya ferina*	1551	KJ710708
Anseriformes	*Aythya fuligula*	1551	KJ722069
Anseriformes	*Branta canadensis*	1551	DQ019124
Anseriformes	*Cairina moschata*	1551	EU755254
Anseriformes	*Cygnus atratus*	1551	FJ379295
Anseriformes	*Cygnus columbianus*	1551	DQ083161
Anseriformes	*Cygnus columbianus bewickii*	1551	JQ282800
Anseriformes	*Cygnus columbianus jankowskii*	1551	KF800698
Anseriformes	*Cygnus cygnus*	1551	KP981363
Anseriformes	*Cygnus olor*	1551	KP981364
Anseriformes	*Dendrocygna javanica*	1551	FJ379296
Anseriformes	*Mareca falcata*	1551	KC759527
Anseriformes	*Mareca strepera*	1551	MN186586

Anseriformes	*Mergus merganser*	1551	KU140667
Anseriformes	*Mergus squamatus*	1551	HQ833701
Anseriformes	*Netta rufina*	1551	KC466568
Anseriformes	*Sibirionetta formosa*	1551	JF730435
Anseriformes	*Tadorna ferruginea*	1551	KF684946
Anseriformes	*Tadorna tadorna*	1551	KU140668
Apodiformes	*Amazilia brevirostris*	1551	KP722043
Apodiformes	*Amazilia millerii*	1551	KP722042
Apodiformes	*Amazilia rondoniae*	1551	KP722041
Apodiformes	*Amazilia versicolor*	1551	KF624601
Apodiformes	*Archilochus colubris*	1551	EF532935
Apodiformes	*Calliphlox amethystina*	1551	KP853095
Apodiformes	*Chaetura pelagica*	1551	KT809406
Apodiformes	*Chrysolampis mosquitus*	1551	KJ619585
Apodiformes	*Cypseloides fumigatus*	1551	KY688216
Apodiformes	*Florisuga fusca*	1551	KP853096
Apodiformes	*Florisuga mellivora*	1551	KJ619588
Apodiformes	*Glaucis hirsutus*	1551	KT265275
Apodiformes	*Heliodoxa aurescens*	1551	KP853094
Apodiformes	*Hylocharis cyanus*	1551	KJ619586
Apodiformes	*Lophornis magnificus*	1551	KT265276
Apodiformes	*Oreotrochilus melanogaster*	1551	KJ619587
Apodiformes	*Phaethornis malaris*	1551	KP853097
Apterygiformes	*Apteryx haastii*	1551	AF338708
Apterygiformes	*Apteryx mantelli*	1542	KU695537
Apterygiformes	*Apteryx owenii*	1551	GU071052
Apterygiformes	*Apteryx rowi*	1551	MN998652
Bucerotiformes	*Aceros waldeni*	1551	HQ834450
Bucerotiformes	*Anthracoceros coronatus*	1551	MF435900

Bucerotiformes	*Buceros bicornis*	1551	MF325011
Bucerotiformes	*Buceros rhinoceros silvestris*	1551	MG596878
Bucerotiformes	*Bucorvus leadbeateri*	1551	HM640209
Bucerotiformes	*Bycanistes brevis*	1551	HM640210
Bucerotiformes	*Penelopides panini*	1551	HQ834451
Bucerotiformes	*Rhyticeros undulatus*	1551	MG171195
Caprimulgiformes	*Aegotheles cristatus*	1551	EU344979
Caprimulgiformes	*Caprimulgus indicus*	1551	KM272749
Casuariiformes	*Casuarius casuarius*	1551	AF338713
Casuariiformes	*Dromaius baudinianus*	1551	MK625178
Casuariiformes	*Dromaius novaehollandiae*	1551	AF338711
Charadriiformes	*Aethia cristatella*	1551	MN337912
Charadriiformes	*Arenaria interpres*	1551	AY074885
Charadriiformes	*Calidris pugnax*	1551	MN956840
Charadriiformes	*Calidris pygmaea*	1551	KY434065
Charadriiformes	*Calidris ruficollis*	1551	MG736926
Charadriiformes	*Calidris tenuirostris*	1551	MK992912
Charadriiformes	*Charadrius alexandrinus*	1551	MF565382
Charadriiformes	*Charadrius placidus*	1551	KY419888
Charadriiformes	*Chroicocephalus ridibundus*	1551	KM577662
Charadriiformes	*Gallinago stenura*	1551	KY888681
Charadriiformes	*Gelochelidon nilotica*	1551	MF582631
Charadriiformes	*Haematopus ater*	1551	AY074886
Charadriiformes	*Haematopus ostralegus*	1551	MH727533
Charadriiformes	*Himantopus himantopus*	1551	KY623656
Charadriiformes	*Hydrophasianus chirurgus*	1551	MH219929
Charadriiformes	*Ichthyaetus relictus*	1551	KC760146
Charadriiformes	*Jacana jacana*	1551	KJ631049
Charadriiformes	*Jacana spinosa*	1551	KJ631048

Charadriiformes	*Larus brunnicephalus*	1551	JX155863
Charadriiformes	*Larus crassirostris*	1551	KM507782
Charadriiformes	*Larus dominicanus*	1551	AY293619
Charadriiformes	*Larus vegae*	1551	KT943749
Charadriiformes	*Limosa lapponica*	1551	MK341549
Charadriiformes	*Limosa lapponica baueri*	1551	KX371106
Charadriiformes	*Numenius phaeopus*	1551	KP308149
Charadriiformes	*Numenius tenuirostris*	1551	MK108195
Charadriiformes	*Phalaropus lobatus*	1551	KY765409
Charadriiformes	*Pinguinus impennis*	1551	KU158188
Charadriiformes	*Pluvialis fulva*	1551	KX639757
Charadriiformes	*Recurvirostra avosetta*	1551	KP757766
Charadriiformes	*Saundersilarus saundersi*	1551	KJ631624
Charadriiformes	*Scolopax rusticola*	1551	KM434134
Charadriiformes	*Stercorarius maccormicki*	1551	KM401546
Charadriiformes	*Sterna hirundo*	1551	MF582632
Charadriiformes	*Sterna paradisaea*	1551	MK946458
Charadriiformes	*Sternula albifrons*	1551	KT350612
Charadriiformes	*Synthliboramphus antiquus*	1551	AP009042
Charadriiformes	*Synthliboramphus wumizusume*	1551	KT592378
Charadriiformes	*Tringa erythropus*	1551	KX230491
Charadriiformes	*Tringa glareola*	1551	KY128485
Charadriiformes	*Tringa guttifer*	1551	MK905885
Charadriiformes	*Tringa nebularia*	1551	MG883743
Charadriiformes	*Tringa ochropus*	1551	KX668223
Charadriiformes	*Tringa semipalmata*	1551	MF036175
Charadriiformes	*Tringa totanus*	1551	MK922124
Charadriiformes	*Vanellus cinereus*	1551	KM873665
Charadriiformes	*Vanellus vanellus*	1551	KM577158

Charadriiformes	*Xenus cinereus*	1551	KX644890
Ciconiiformes	*Ciconia boyciana*	1551	AB026193
Ciconiiformes	*Ciconia ciconia*	1551	AB026818
Ciconiiformes	*Ciconia nigra*	1551	KY767670
Columbiformes	*Alopecoenas salamonis*	1551	KX902250
Columbiformes	*Caloenas maculata*	1551	KX902249
Columbiformes	*Caloenas nicobarica*	1551	MG590264
Columbiformes	*Columba hodgsonii*	1551	MN919176
Columbiformes	*Columba jouyi*	1551	KX902247
Columbiformes	*Columba livia*	1551	KP319029
Columbiformes	*Columba rupestris*	1551	KX902246
Columbiformes	*Didunculus strigirostris*	1551	MG590266
Columbiformes	*Ectopistes migratorius*	1551	KC489473
Columbiformes	*Geopelia cuneata*	1551	MN930521
Columbiformes	*Geopelia striata*	1551	MG590276
Columbiformes	*Geotrygon violacea*	1551	HM640213
Columbiformes	*Goura cristata*	1551	MG590271
Columbiformes	*Goura cristata minor*	1560	LN589994
Columbiformes	*Goura scheepmakeri*	1551	MG590282
Columbiformes	*Goura sclaterii*	1551	MG590288
Columbiformes	*Goura victoria*	1551	MG590296
Columbiformes	*Goura victoria beccarii*	1560	LN589993
Columbiformes	*Hemiphaga novaeseelandiae*	1551	EU725864
Columbiformes	*Leptotila verreauxi*	1551	HM640214
Columbiformes	*Otidiphaps nobilis*	1551	MG590265
Columbiformes	*Patagioenas fasciata monilis*	1551	KX902239
Columbiformes	*Raphus cucullatus*	1551	KX902236
Columbiformes	*Streptopelia chinensis*	1551	KP273832
Columbiformes	*Streptopelia decaocto*	1551	KY827036

Columbiformes	*Streptopelia orientalis*	1551	KT182929
Columbiformes	*Trugon terrestris*	1551	MG590263
Columbiformes	*Zenaida auriculata*	1551	HM640211
Columbiformes	*Zenaida macroura*	1551	KX902235
Coraciiformes	*Alcedo atthis*	1551	KY964271
Coraciiformes	*Ceryle rudis*	1551	KJ461938
Coraciiformes	*Eurystomus orientalis*	1551	EU344978
Coraciiformes	*Halcyon coromanda*	1551	KT356219
Coraciiformes	*Halcyon pileata*	1551	KJ476742
Coraciiformes	*Halcyon smyrnensis*	1551	KY940559
Coraciiformes	*Megaceryle lugubris*	1551	KY940558
Coraciiformes	*Merops viridis*	1551	KU821702
Coraciiformes	*Todiramphus sanctus vagans*	1551	EU410489
Cuculiformes	*Cuculus canorus*	1551	MN067867
Cuculiformes	*Cuculus poliocephalus*	1551	KT378620
Cuculiformes	*Geococcyx californianus*	1551	HM640212
Cuculiformes	*Urodynamis taitensis*	1551	EU410487
Dinornithiformes	*Anomalopteryx didiformis*	1551	MK778441
Dinornithiformes	*Dinornis giganteus*	1551	AY016013
Dinornithiformes	*Emeus crassus*	1551	AY016015
Falconiformes	*Caracara cheriway*	1551	MN231451
Falconiformes	*Caracara creightoni*	1551	MN231452
Falconiformes	*Caracara plancus*	1551	MN231450
Falconiformes	*Falco amurensis*	1551	KX987839
Falconiformes	*Falco cherrug*	1551	KP337902
Falconiformes	*Falco columbarius*	1551	KM264304
Falconiformes	*Falco naumanni*	1551	KM251414
Falconiformes	*Falco peregrinus*	1551	JQ282801
Falconiformes	*Falco rusticolus*	1551	KT989235

Falconiformes	*Falco sparverius*	1551	DQ780880
Falconiformes	*Falco tinnunculus*	1551	EU196361
Falconiformes	*Micrastur gilvicollis*	1551	DQ780881
Falconiformes	*Phalcoboenus australis*	1551	KP064202
Galliformes	*Acryllium vulturinum*	1551	FJ752436
Galliformes	*Alectoris chukar*	1551	KY829450
Galliformes	*Alectura lathami*	1551	AY346091
Galliformes	*Arborophila ardens*	1551	KJ716444
Galliformes	*Arborophila atrogularis*	1551	MN868239
Galliformes	*Arborophila brunneopectus*	1551	KC352730
Galliformes	*Arborophila cambodiana*	1551	MN868241
Galliformes	*Arborophila crudigularis*	1551	MN868242
Galliformes	*Arborophila gingica*	1551	FJ752425
Galliformes	*Arborophila hyperythra*	1551	MN868244
Galliformes	*Arborophila javanica*	1551	MN868245
Galliformes	*Arborophila mandellii*	1551	MN868246
Galliformes	*Arborophila orientalis*	1551	MN868247
Galliformes	*Arborophila rubrirostris*	1551	MN868248
Galliformes	*Arborophila rufipectus*	1551	FJ194942
Galliformes	*Arborophila rufogularis*	1551	FJ752424
Galliformes	*Arborophila torqueola*	1551	MN868249
Galliformes	*Bambusicola fytchii*	1551	FJ752423
Galliformes	*Bambusicola thoracicus*	1551	EU165706
Galliformes	*Callipepla squamata*	1551	KT722338
Galliformes	*Caloperdix oculeus*	1551	KJ914546
Galliformes	*Chrysolophus amherstiae*	1551	FJ752434
Galliformes	*Chrysolophus pictus*	1551	MN857545
Galliformes	*Colinus virginianus*	1551	KJ914548
Galliformes	*Coturnix chinensis*	1551	AB073301

Galliformes	*Coturnix japonica*	1551	KX712089
Galliformes	*Crax daubentoni*	1551	KJ914544
Galliformes	*Crax rubra*	1551	KJ914545
Galliformes	*Crossoptilon auritum*	1551	JF937589
Galliformes	*Crossoptilon crossoptilon*	1551	HQ891119
Galliformes	*Crossoptilon harmani*	1551	KP259806
Galliformes	*Crossoptilon mantchuricum*	1551	KP259807
Galliformes	*Francolinus pintadeanus*	1551	EU165707
Galliformes	*Gallus gallus*	1551	HQ857211
Galliformes	*Gallus gallus bankiva*	1551	AP003323
Galliformes	*Gallus gallus gallus*	1551	MN013407
Galliformes	*Gallus gallus jabouillei*	1551	GU261696
Galliformes	*Gallus gallus murghi*	1551	GU261708
Galliformes	*Gallus gallus spadiceus*	1551	GU261690
Galliformes	*Gallus lafayetii*	1551	AP003325
Galliformes	*Gallus sonneratii*	1551	AP006741
Galliformes	*Gallus varius*	1551	AP003324
Galliformes	*Haematortyx sanguiniceps*	1551	KY411591
Galliformes	*Ithaginis cruentus*	1551	KY411592
Galliformes	*Lagopus lagopus*	1551	KX609784
Galliformes	*Lagopus muta*	1551	KX609785
Galliformes	*Lagopus muta japonica*	1551	LC528834
Galliformes	*Lophophorus impejanus*	1551	MF975712
Galliformes	*Lophophorus lhuysii*	1551	GQ871234
Galliformes	*Lophophorus sclateri*	1551	FJ752432
Galliformes	*Lophura ignita*	1551	AB164627
Galliformes	*Lophura nycthemera*	1551	EU417810
Galliformes	*Lophura swinhoii*	1551	KF218954
Galliformes	*Lyrurus tetrix*	1551	KF955638

Galliformes	*Meleagris gallopavo*	1551	JF275060
Galliformes	*Numida meleagris*	1551	KY865420
Galliformes	*Pavo cristatus*	1551	KF444060
Galliformes	*Pavo muticus*	1551	EU417811
Galliformes	*Perdix dauurica*	1551	KY411596
Galliformes	*Perdix hodgsoniae*	1551	KF027440
Galliformes	*Perdix perdix*	1551	KX838508
Galliformes	*Phasianus colchicus*	1551	JF739859
Galliformes	*Phasianus colchicus alaschanicus*	1551	KU049722
Galliformes	*Phasianus versicolor*	1551	AB164626
Galliformes	*Pipile pipile*	1551	KU221053
Galliformes	*Polyplectron bicalcaratum*	1551	EU417812
Galliformes	*Polyplectron germaini*	1551	KF422893
Galliformes	*Polyplectron malacense*	1551	MN240360
Galliformes	*Polyplectron napoleonis*	1551	KJ939353
Galliformes	*Ptilopachus petrosus*	1551	KJ914543
Galliformes	*Pucrasia macrolopha*	1551	FJ752429
Galliformes	*Syrmaticus ellioti*	1551	AB164624
Galliformes	*Syrmaticus humiae*	1551	AB164625
Galliformes	*Syrmaticus reevesii*	1551	AB164623
Galliformes	*Syrmaticus soemmerringii ijimae*	1551	AB164622
Galliformes	*Tetrao parvirostris kamtschaticus*	1551	MK820677
Galliformes	*Tetrao parvirostris parvirostris*	1551	MK820678
Galliformes	*Tetrao urogallus aquitanicus*	1551	MG583885
Galliformes	*Tetraogallus himalayensis*	1551	KR349185
Galliformes	*Tetraogallus tibetanus*	1551	KY766921
Galliformes	*Tetraophasis obscurus*	1551	JF921876
Galliformes	*Tetraophasis szechenyii*	1551	FJ752428
Galliformes	*Tetrastes bonasia*	1551	FJ752435

Galliformes	*Tetrastes sewerzowi*	1551	KJ997914
Galliformes	*Tragopan caboti*	1551	GU187969
Galliformes	*Tragopan temminckii*	1551	FJ752427
Gaviiformes	*Gavia arctica*	1551	MH064399
Gaviiformes	*Gavia pacifica*	1551	MK342599
Gaviiformes	*Gavia stellata*	1551	AY293618
Gruiformes	*Amaurornis akool*	1551	KJ192198
Gruiformes	*Amaurornis phoenicurus*	1551	KJ874440
Gruiformes	*Anthropoides paradiseus*	1554	MH041488
Gruiformes	*Anthropoides virgo*	1551	FJ769845
Gruiformes	*Antigone antigone*	1551	FJ769854
Gruiformes	*Antigone canadensis*	1551	FJ769855
Gruiformes	*Antigone rubicunda*	1551	FJ769853
Gruiformes	*Antigone vipio*	1551	FJ769852
Gruiformes	*Atlantisia rogersi*	1551	MH029238
Gruiformes	*Balearica pavonina*	1551	FJ769842
Gruiformes	*Balearica regulorum*	1551	FJ769841
Gruiformes	*Coturnicops exquisitus*	1551	AP010823
Gruiformes	*Eulabeornis castaneoventris*	1551	KF644583
Gruiformes	*Fulica atra*	1551	KP313718
Gruiformes	*Gallicrex cinerea*	1551	KP057881
Gruiformes	*Gallinula chloropus*	1548	HQ896036
Gruiformes	*Gallirallus australis australis*	1551	KF425525
Gruiformes	*Gallirallus australis hectori*	1551	KF701060
Gruiformes	*Gallirallus okinawae*	1551	AP010821
Gruiformes	*Gallirallus philippensis*	1551	KF701061
Gruiformes	*Gallirallus striatus*	1551	MH219930
Gruiformes	*Grus americana*	1551	FJ769848
Gruiformes	*Grus carunculatus*	1551	MH041486

Gruiformes	*Grus grus*	1551	FJ769849
Gruiformes	*Grus japonensis*	1551	MH041485
Gruiformes	*Grus leucogeranus*	1551	MH041490
Gruiformes	*Grus monacha*	1551	FJ769850
Gruiformes	*Grus nigricollis*	1551	FJ769851
Gruiformes	*Heliornis fulica*	1548	KF644581
Gruiformes	*Lewinia muelleri*	1551	KF644584
Gruiformes	*Otis tarda*	1551	FJ751803
Gruiformes	*Porphyrio hochstetteri*	1551	EF532934
Gruiformes	*Porphyrio porphyrio*	1551	KF701062
Gruiformes	*Porzana fusca*	1551	KY009736
Gruiformes	*Porzana paykullii*	1551	MG200164
Gruiformes	*Porzana pusilla*	1551	KY009737
Gruiformes	*Rallina eurizonoides sepiaria*	1551	AP010822
Gruiformes	*Rallus aquaticus*	1551	MH229988
Gruiformes	*Rhynochetos jubatus*	1551	EF532933
Gruiformes	*Sarothrura ayresi*	1551	KY075897
Passeriformes	*Abrornis inornata*	1551	KF742677
Passeriformes	*Abrornis proregulus*	1551	MG189603
Passeriformes	*Acanthis flammea*	1551	KR422696
Passeriformes	*Acanthiza nana*	1551	KY994589
Passeriformes	*Acridotheres cristatellus*	1551	JF810423
Passeriformes	*Acridotheres tristis*	1551	HQ915864
Passeriformes	*Acrocephalus orientalis*	1551	MG681103
Passeriformes	*Acrocephalus scirpaceus*	1551	AM889139
Passeriformes	*Aegithalos bonvaloti*	1551	KF951087
Passeriformes	*Aegithalos caudatus caudatus*	1551	KF951088
Passeriformes	*Aegithalos concinnus concinnus*	1551	KF951091
Passeriformes	*Aegithalos concinnus talifuensis*	1551	KF951092

Passeriformes	*Aegithalos fuliginosus*	1551	KF951086
Passeriformes	*Aegithalos glaucogularis*	1551	KF951089
Passeriformes	*Aegithalos glaucogularis vinaceus*	1551	KF951090
Passeriformes	*Aethopyga gouldiae*	1551	KP772257
Passeriformes	*Agelaius phoeniceus*	1551	KM078767
Passeriformes	*Agelaius phoeniceus*	1551	JX516062
Passeriformes	*Akialoa obscura*	1551	KU158190
Passeriformes	*Alauda arvensis*	1551	JQ322641
Passeriformes	*Alaudala cheleensis*	1551	MG681104
Passeriformes	*Alcippe morrisonia hueti*	1551	KX376475
Passeriformes	*Amblyramphus holosericeus*	1551	JX516063
Passeriformes	*Anthus novaeseelandiae*	1551	KC545397
Passeriformes	*Anthus richardi*	1551	MH593382
Passeriformes	*Arremon aurantiirostris*	1551	KR780063
Passeriformes	*Artamus cinereus*	1551	MF784400
Passeriformes	*Artamus cyanopterus*	1551	KY994613
Passeriformes	*Calandrella cinerea*	1551	MH061201
Passeriformes	*Callaeas cinereus*	1551	KU158191
Passeriformes	*Calliope calliope*	1551	HQ690246
Passeriformes	*Campylorhynchus brunneicapillus*	1551	KU057376
Passeriformes	*Campylorhynchus zonatus*	1560	KF509924
Passeriformes	*Cardellina canadensis*	1551	MK033135
Passeriformes	*Cardinalis cardinalis*	1551	MH700631
Passeriformes	*Carduelis carduelis*	1551	KM078790
Passeriformes	*Carpodacus erythrinus*	1551	MN122832
Passeriformes	*Carpodacus roseus*	1551	KM078779
Passeriformes	*Carpodacus rubicilloides*	1551	MH363715
Passeriformes	*Castanozoster thoracicus*	1551	KT272188
Passeriformes	*Cecropis daurica*	1551	KJ499911

Passeriformes	*Chloris sinica*	1551	MH102388
Passeriformes	*Chloris sinica ussuriensis*	1551	MH047559
Passeriformes	*Chlorophanes spiza*	1551	KM078778
Passeriformes	*Chrysomus cyanopus*	1551	JX516076
Passeriformes	*Chrysomus icterocephalus*	1551	JX516060
Passeriformes	*Chrysomus ruficapillus*	1551	JX516056
Passeriformes	*Chrysomus thilius*	1551	JX516069
Passeriformes	*Chrysomus xanthophthalmus*	1551	JX516059
Passeriformes	*Climacteris picumnus*	1554	KY994598
Passeriformes	*Cnemotriccus fuscatus*	1551	AY596278
Passeriformes	*Coccothraustes coccothraustes*	1557	KM078789
Passeriformes	*Colluricincla harmonica*	1551	KY994593
Passeriformes	*Copsychus saularis*	1551	KU058637
Passeriformes	*Cormobates leucophaeus*	1551	KY994580
Passeriformes	*Corvus brachyrhynchos*	1551	KP403809
Passeriformes	*Corvus corax*	1551	MN122895
Passeriformes	*Corvus corone orientalis*	1551	MK714020
Passeriformes	*Corvus coronoides*	1542	MF370524
Passeriformes	*Corvus cryptoleucus*	1551	KX245139
Passeriformes	*Corvus dauuricus*	1551	MN735458
Passeriformes	*Corvus hawaiiensis*	1551	KP161620
Passeriformes	*Corvus macrorhynchos*	1551	KR072661
Passeriformes	*Corvus macrorhynchos intermedius*	1542	MN069302
Passeriformes	*Corvus moriorum*	1551	KX822153
Passeriformes	*Corvus pectoralis*	1551	MN310552
Passeriformes	*Corvus splendens*	1551	KP019939
Passeriformes	*Culicicapa ceylonensis*	1557	MH880820
Passeriformes	*Curaeus curaeus*	1551	JX516070
Passeriformes	*Cyanistes cyanus*	1551	KX388472

Passeriformes	*Cyanoderma ruficeps*	1551	KU362930
Passeriformes	*Cyanopica cyanus*	1551	JN108020
Passeriformes	*Cyanopica cyanus koreensis*	1551	KT934323
Passeriformes	*Cyanoptila cyanomelana*	1551	HQ896033
Passeriformes	*Daphoenositta chrysoptera*	1551	KY994604
Passeriformes	*Dendrocitta formosae*	1551	MK875763
Passeriformes	*Dicrurus hottentottus*	1551	MK814795
Passeriformes	*Dives dives*	1551	JX516061
Passeriformes	*Emberiza chrysophrys*	1548	HQ896034
Passeriformes	*Emberiza cioides*	1551	KF322027
Passeriformes	*Emberiza fucata*	1551	KT737824
Passeriformes	*Emberiza jankowskii*	1551	KP738714
Passeriformes	*Emberiza leucocephalos*	1551	KY349100
Passeriformes	*Emberiza pusilla*	1551	KC407232
Passeriformes	*Emberiza rutila*	1551	KC952874
Passeriformes	*Emberiza sulphurata*	1551	KY419885
Passeriformes	*Emberiza tristrami*	1551	HQ896035
Passeriformes	*Eophona migratoria*	1551	KX423959
Passeriformes	*Eophona personata*	1551	KX812499
Passeriformes	*Eopsaltria australis*	1551	KM374659
Passeriformes	*Eopsaltria georgiana*	1551	KM374635
Passeriformes	*Eopsaltria griseogularis*	1551	KM374625
Passeriformes	*Epthianura albifrons*	1551	JX901072
Passeriformes	*Eremophila alpestris*	1551	MH061202
Passeriformes	*Eremopsaltria mongolica*	1551	KM078792
Passeriformes	*Erythrogenys gravivox*	1551	MH737742
Passeriformes	*Euphagus cyanocephalus*	1551	JX516072
Passeriformes	*Ficedula albicilla*	1551	MN125374
Passeriformes	*Ficedula albicollis*	1560	KF293721

Passeriformes	*Ficedula zanthopygia*	1551	JN018411
Passeriformes	*Fringilla coelebs*	1551	KM078769
Passeriformes	*Fringilla montifringilla*	1551	JQ922259
Passeriformes	*Fringilla polatzeki*	1551	KU705764
Passeriformes	*Fringilla teydea teydea*	1551	KU705760
Passeriformes	*Fulvetta ruficapilla*	1551	MK988446
Passeriformes	*Garrulax albogularis*	1551	KX082660
Passeriformes	*Garrulax canorus*	1551	KT633399
Passeriformes	*Garrulax cineraceus*	1551	KF926988
Passeriformes	*Garrulax formosus*	1551	KR020504
Passeriformes	*Garrulax ocellatus*	1551	KP995195
Passeriformes	*Garrulax perspicillatus*	1551	KF997865
Passeriformes	*Garrulax poecilorhynchus*	1551	KR909134
Passeriformes	*Garrulax sannio*	1551	KT373847
Passeriformes	*Garrulus glandarius*	1551	JN018413
Passeriformes	*Geokichla sibirica sibirica*	1542	MK377247
Passeriformes	*Geospiza magnirostris*	1560	MG682351
Passeriformes	*Gerygone igata*	1551	KC545399
Passeriformes	*Gnorimopsar chopi*	1551	JX516055
Passeriformes	*Gracula religiosa*	1551	JF937590
Passeriformes	*Gymnomystax mexicanus*	1551	JX516075
Passeriformes	*Haemorhous cassinii*	1551	KM078786
Passeriformes	*Haemorhous mexicanus*	1551	KM078782
Passeriformes	*Hemignathus flavus*	1551	KM078780
Passeriformes	*Hemignathus parvus*	1551	KM078799
Passeriformes	*Hemignathus stejnegeri*	1551	KM078801
Passeriformes	*Hemignathus virens virens*	1551	KM078788
Passeriformes	*Henicorhina leucosticta*	1551	KJ746107
Passeriformes	*Hesperiphona vespertina*	1551	KM078770

Passeriformes	*Heteralocha acutirostris*	1542	KU158193
Passeriformes	*Heterophasia melanoleuca*	1551	MK408609
Passeriformes	*Himatione sanguinea*	1551	KM078773
Passeriformes	*Hirundo rustica erythrogaster*	1557	KX398931
Passeriformes	*Hirundo rustica gutturalis*	1557	KP148840
Passeriformes	*Hirundo tahitica*	1557	MN849306
Passeriformes	*Horornis fortipes*	1551	MK051002
Passeriformes	*Ixos mcclellandii*	1551	KX640824
Passeriformes	*Lalage tricolor*	1551	KY994597
Passeriformes	*Lanius collurio*	1551	MN122863
Passeriformes	*Lanius cristatus*	1551	KT004451
Passeriformes	*Lanius isabellinus*	1551	KP995437
Passeriformes	*Lanius schach*	1551	KU058639
Passeriformes	*Lanius sphenocercus sphenocercus*	1551	KU884610
Passeriformes	*Lanius tephronotus*	1551	JX486029
Passeriformes	*Lanius tigrinus*	1551	LC428205
Passeriformes	*Leiothrix argentauris*	1548	HQ690245
Passeriformes	*Leiothrix lutea*	1551	JQ423933
Passeriformes	*Lepidocolaptes angustirostris*	1551	KY628989
Passeriformes	*Leucosticte arctoa*	1551	KM078791
Passeriformes	*Leucosticte brandti*	1551	KM078775
Passeriformes	*Liocichla omeiensis*	1551	KU886092
Passeriformes	*Locustella castanea*	1551	MN597062
Passeriformes	*Locustella pryeri*	1551	KJ001760
Passeriformes	*Lonchura caniceps*	1551	MF770314
Passeriformes	*Lonchura castaneothorax*	1551	MF770348
Passeriformes	*Lonchura flaviprymna*	1551	MF770379
Passeriformes	*Lonchura forbesi*	1551	MF770384
Passeriformes	*Lonchura grandis*	1551	MF770399

Passeriformes	*Lonchura grandis x Lonchura castaneothorax*	1551	MF770420
Passeriformes	*Lonchura hunsteini*	1551	MF770414
Passeriformes	*Lonchura leucosticta*	1551	MF770311
Passeriformes	*Lonchura melaena*	1551	MF770428
Passeriformes	*Lonchura nevermanni*	1551	MF770442
Passeriformes	*Lonchura nigerrima*	1551	MF770452
Passeriformes	*Lonchura punctulata*	1551	KR184724
Passeriformes	*Lonchura spectabilis*	1551	MF770464
Passeriformes	*Lonchura striata*	1551	KR080134
Passeriformes	*Lonchura stygia*	1551	MF770484
Passeriformes	*Lophophanes dichrous*	1551	KX388477
Passeriformes	*Loxia curvirostra*	1551	KM078800
Passeriformes	*Loxops caeruleirostris*	1551	KM078776
Passeriformes	*Loxops coccineus*	1551	KM078785
Passeriformes	*Loxops mana*	1551	KM078768
Passeriformes	*Luscinia cyanura*	1551	KF997864
Passeriformes	*Machlolophus spilonotus*	1551	KX388476
Passeriformes	*Macroagelaius imthurni*	1551	JX516073
Passeriformes	*Manorina melanocephala*	1551	KY994587
Passeriformes	*Melamprosops phaeosoma*	1551	KM078793
Passeriformes	*Melanocorypha mongolica*	1551	KY887027
Passeriformes	*Melophus lathami*	1551	KX702277
Passeriformes	*Menura novaehollandiae*	1551	AY542313
Passeriformes	*Microspingus cabanisi*	1551	KT272189
Passeriformes	*Microspingus lateralis*	1551	KT272190
Passeriformes	*Minla ignotincta*	1551	KT995474
Passeriformes	*Mionectes oleagineus*	1551	KJ742591
Passeriformes	*Moho braccatus*	1551	KU158189

Passeriformes	*Molothrus aeneus*	1551	JX516067
Passeriformes	*Molothrus badius*	1551	JX516074
Passeriformes	*Monticola gularis*	1551	KX506858
Passeriformes	*Montifringilla adamsi*	1551	KJ148630
Passeriformes	*Montifringilla henrici*	1551	MH049432
Passeriformes	*Montifringilla nivalis*	1551	KJ148628
Passeriformes	*Montifringilla ruficollis*	1551	KC836121
Passeriformes	*Montifringilla taczanowskii*	1551	KJ148631
Passeriformes	*Motacilla alba*	1551	KT736087
Passeriformes	*Motacilla cinerea*	1551	KR092187
Passeriformes	*Motacilla lugens*	1551	KU246035
Passeriformes	*Motacilla tschutschensis*	1551	MN217252
Passeriformes	*Muscicapa griseisticta*	1551	MK390479
Passeriformes	*Muscicapa latirostris*	1551	MK770602
Passeriformes	*Muscicapa sibirica*	1551	MK770601
Passeriformes	*Myadestes myadestinus*	1551	KU158194
Passeriformes	*Napothera epilepidota*	1551	KX831093
Passeriformes	*Nesopsar nigerrimus*	1551	JX516054
Passeriformes	*Nesoptilotis leucotis*	1551	KY994594
Passeriformes	*Niltava davidi*	1551	KY024217
Passeriformes	*Notiomystis cincta*	1551	KC545400
Passeriformes	*Nucifraga columbiana*	1551	KF509923
Passeriformes	*Oedistoma iliolophus*	1551	KJ909186
Passeriformes	*Oenanthe isabellina*	1551	KU097327
Passeriformes	*Oenanthe oenanthe*	1551	MT431106
Passeriformes	*Oreomystis bairdi*	1551	KM078807
Passeriformes	*Oreopsar bolivianus*	1551	JX516058
Passeriformes	*Oriolus chinensis*	1551	JQ083495
Passeriformes	*Pachycephala melanura*	1551	KY994595

Passeriformes	*Pachycephala occidentalis*	1551	KY994609
Passeriformes	*Pachycephala pectoralis*	1551	KY994581
Passeriformes	*Padda oryzivora*	1551	KT633398
Passeriformes	*Paradoxornis fulvifrons*	1551	KT598466
Passeriformes	*Paradoxornis heudei*	1551	MN865117
Passeriformes	*Paradoxornis webbianus*	1551	KF725775
Passeriformes	*Pardaliparus venustulus*	1551	KP313823
Passeriformes	*Pardalotus punctatus*	1551	KY994592
Passeriformes	*Pardalotus striatus*	1551	KY994585
Passeriformes	*Paroreomyza montana*	1551	KM078771
Passeriformes	*Parus major*	1551	MH638304
Passeriformes	*Parus major*	1551	KP137624
Passeriformes	*Parus monticolus*	1551	KX388474
Passeriformes	*Passer ammodendri*	1551	KT895996
Passeriformes	*Passer domesticus*	1551	KM078784
Passeriformes	*Passer montanus*	1551	MH211397
Passeriformes	*Passer montanus*	1551	JX486030
Passeriformes	*Passer montanus saturatus*	1551	KM577704
Passeriformes	*Pericrocotus ethologus*	1551	JX256246
Passeriformes	*Periparus ater*	1551	KM588075
Passeriformes	*Petroica australis*	1551	KC545401
Passeriformes	*Petroica boodang*	1551	JX901074
Passeriformes	*Petroica goodenovii*	1551	JX901075
Passeriformes	*Petroica macrocephala*	1551	KC545402
Passeriformes	*Petroica multicolor*	1551	MT181966
Passeriformes	*Petroica phoenicea*	1551	JX901076
Passeriformes	*Petronia petronia*	1551	MF071218
Passeriformes	*Philesturnus carunculatus*	1551	KC545403
Passeriformes	*Phoenicurus auroreus*	1551	KF997863

Passeriformes	*Phylloscopus borealoides*	1551	MN125373
Passeriformes	*Phylloscopus fuscatus*	1551	MG681101
Passeriformes	*Phylloscopus occisinensis*	1551	MK513447
Passeriformes	*Pica pica*	1551	HQ915867
Passeriformes	*Pinicola enucleator*	1551	KM078781
Passeriformes	*Piranga bidentata*	1551	MH700647
Passeriformes	*Piranga erythrocephala*	1551	MH700649
Passeriformes	*Piranga flava*	1551	MH700637
Passeriformes	*Piranga hepatica*	1551	MH700640
Passeriformes	*Piranga leucoptera*	1551	MH700653
Passeriformes	*Piranga ludoviciana*	1551	MH700645
Passeriformes	*Piranga lutea*	1551	MH700639
Passeriformes	*Piranga olivacea*	1551	MH700642
Passeriformes	*Piranga roseogularis*	1551	MH700633
Passeriformes	*Piranga rubra*	1551	MH700635
Passeriformes	*Piranga rubriceps*	1551	MH700651
Passeriformes	*Pitta nympha*	1530	KJ680302
Passeriformes	*Podoces hendersoni*	1551	GU592504
Passeriformes	*Poecile atricapillus*	1551	KJ909190
Passeriformes	*Poecile montanus*	1551	MN122849
Passeriformes	*Poecile montanus*	1551	KX388478
Passeriformes	*Poecile montanus baicalensis*	1551	KX388479
Passeriformes	*Poecile palustris*	1560	KP184518
Passeriformes	*Pomatorhinus ruficollis*	1551	KT970675
Passeriformes	*Poodytes punctatus*	1551	KC545398
Passeriformes	*Progne chalybea*	1551	JQ071623
Passeriformes	*Prosthemadera novaeseelandiae*	1551	KC545404
Passeriformes	*Prunella fulvescens*	1551	KY471556
Passeriformes	*Prunella montanella*	1551	KR422695

Passeriformes	*Prunella strophiata*	1551	KU975800
Passeriformes	*Pseudoleistes guirahuro*	1551	JX516071
Passeriformes	*Pseudoleistes virescens*	1551	JX516066
Passeriformes	*Pseudonestor xanthophrys*	1551	KM078809
Passeriformes	*Pseudopodoces humilis*	1551	HM535648
Passeriformes	*Psittiparus gularis*	1551	KX397391
Passeriformes	*Psittirostra psittacea*	1551	KU158196
Passeriformes	*Ptilotula penicillata*	1551	KY994588
Passeriformes	*Pycnonotus melanicterus*	1551	KJ186975
Passeriformes	*Pycnonotus sinensis*	1551	GU475148
Passeriformes	*Pycnonotus sinensis hainanus*	1551	KJ147475
Passeriformes	*Pycnonotus taivanus*	1551	FJ378536
Passeriformes	*Pycnonotus xanthorrhous*	1551	KX129905
Passeriformes	*Pyrgilauda blanfordi*	1551	KJ148629
Passeriformes	*Pyrgilauda davidiana*	1551	KJ148632
Passeriformes	*Pyrrhocorax graculus*	1551	KJ598623
Passeriformes	*Pyrrhocorax pyrrhocorax*	1551	KJ598622
Passeriformes	*Pyrrhula pyrrhula*	1551	KM078804
Passeriformes	*Quiscalus quiscula*	1551	JX516064
Passeriformes	*Regulus calendula*	1551	KJ909188
Passeriformes	*Regulus regulus*	1551	KT934324
Passeriformes	*Remiz consobrinus*	1551	KC463856
Passeriformes	*Rhagologus leucostigma*	1551	MF784399
Passeriformes	*Rhipidura fuliginosa*	1551	KC545405
Passeriformes	*Rupicola peruvianus*	1551	MN602289
Passeriformes	*Saltator similis*	1551	MK419316
Passeriformes	*Schoeniclus aureolus*	1551	KF111713
Passeriformes	*Schoeniclus elegans*	1551	KY349099
Passeriformes	*Schoeniclus rusticus*	1551	KC831775

Passeriformes	*Schoeniclus siemsseni*	1551	KX809695
Passeriformes	*Schoeniclus spodocephala*	1551	KC758647
Passeriformes	*Seicercus borealis*	1551	MK390476
Passeriformes	*Seicercus burkii*	1551	KX977449
Passeriformes	*Seicercus tenellipes*	1551	MK390475
Passeriformes	*Serinus albogularis*	1551	KM078764
Passeriformes	*Serinus canaria*	1551	KM078794
Passeriformes	*Serinus dorsostriatus*	1551	KM078798
Passeriformes	*Sicalis olivascens*	1551	KY628988
Passeriformes	*Sitta carolinensis*	1551	KJ909195
Passeriformes	*Sitta himalayensis*	1551	MK343426
Passeriformes	*Sitta nagaensis*	1551	MK343427
Passeriformes	*Siva cyanouroptera*	1551	MK779708
Passeriformes	*Smicrornis brevirostris*	1551	KY994606
Passeriformes	*Smithornis sharpei*	1551	AF090340
Passeriformes	*Spinus psaltria*	1551	KM078806
Passeriformes	*Spinus spinus*	1551	HQ915866
Passeriformes	*Spizixos semitorques*	1551	KJ174511
Passeriformes	*Sporophila maximiliani*	1551	MF327582
Passeriformes	*Sturnus cineraceus*	1551	HQ896037
Passeriformes	*Sturnus nigricollis*	1551	JQ003192
Passeriformes	*Sturnus sericeus*	1551	HM859900
Passeriformes	*Sturnus vulgaris*	1551	KT946692
Passeriformes	*Suthora nipalensis*	1551	KT598467
Passeriformes	*Sylvia atricapilla*	1551	AM889140
Passeriformes	*Sylvia crassirostris*	1551	AM889141
Passeriformes	*Sylvia curruca*	1551	MG681102
Passeriformes	*Sylviparus modestus*	1551	KP642167
Passeriformes	*Tachycineta albilinea*	1551	JQ071619

Passeriformes	*Tachycineta albiventer*	1551	JQ071620
Passeriformes	*Tachycineta bicolor*	1551	JQ071614
Passeriformes	*Tachycineta cyaneoviridis*	1551	JQ071617
Passeriformes	*Tachycineta euchrysea*	1551	JQ071616
Passeriformes	*Tachycineta leucorrhoa*	1551	JQ071621
Passeriformes	*Tachycineta meyeni*	1551	JQ071622
Passeriformes	*Tachycineta stolzmanni*	1551	JQ071618
Passeriformes	*Tachycineta thalassina*	1551	JQ071615
Passeriformes	*Taeniopygia guttata*	1551	DQ453514
Passeriformes	*Tangara episcopus*	1551	KM078765
Passeriformes	*Terpsiphone atrocaudata*	1551	KT901458
Passeriformes	*Terpsiphone paradisi*	1551	MT554195
Passeriformes	*Tregellasia capito*	1551	KM374637
Passeriformes	*Tregellasia leucops*	1551	KJ909197
Passeriformes	*Trochalopteron affine*	1551	KT182082
Passeriformes	*Trochalopteron elliotii*	1551	KT272404
Passeriformes	*Trochalopteron milnei*	1551	MH238447
Passeriformes	*Turdoides affinis*	1551	MN848144
Passeriformes	*Turdus cardis*	1551	MN865118
Passeriformes	*Turdus eunomus*	1551	KM015261
Passeriformes	*Turdus hortulorum*	1560	KF926987
Passeriformes	*Turdus kessleri*	1551	MG912943
Passeriformes	*Turdus merula*	1560	MN122910
Passeriformes	*Turdus migratorius*	1551	KJ909198
Passeriformes	*Turdus naumanni naumanni*	1551	KJ834096
Passeriformes	*Turdus philomelos*	1551	KC545406
Passeriformes	*Turdus rufiventris*	1542	KT346357
Passeriformes	*Turnagra capensis*	1551	KT894672
Passeriformes	*Uragus sibiricus*	1551	KM078763

Passeriformes	*Urocissa caerulea*	1551	MG932654
Passeriformes	*Urocissa erythrorhyncha*	1551	JQ423932
Passeriformes	*Vestiaria coccinea*	1551	KM078797
Passeriformes	*Vidua chalybeata*	1551	AF090341
Passeriformes	*Vireo olivaceus*	1551	KJ909193
Passeriformes	*Xanthopsar flavus*	1551	JX516065
Passeriformes	*Yuhina diademata*	1551	KT783535
Passeriformes	*Yuhina nigrimenta*	1551	MH916608
Passeriformes	*Zoothera aurea*	1560	KT340629
Passeriformes	*Zosterops abyssinicus*	1551	KX181885
Passeriformes	*Zosterops borbonicus*	1551	MK529728
Passeriformes	*Zosterops erythropleurus*	1551	KT194322
Passeriformes	*Zosterops lateralis*	1551	KC545407
Passeriformes	*Zosterops pallidus*	1551	MK524996
Passeriformes	*Zosterops poliogastrus*	1551	KX181886
Pelecaniformes	*Anhinga rufa*	1551	GU071055
Pelecaniformes	*Ardea cinerea*	1551	KJ190947
Pelecaniformes	*Ardea insignis*	1551	MH737740
Pelecaniformes	*Ardea intermedia*	1551	KX592585
Pelecaniformes	*Ardea modesta*	1551	KJ190944
Pelecaniformes	*Ardea novaehollandiae*	1551	DQ780878
Pelecaniformes	*Ardea purpurea*	1551	KJ190948
Pelecaniformes	*Ardeola bacchus*	1551	KJ190952
Pelecaniformes	*Botaurus stellaris*	1551	KJ190955
Pelecaniformes	*Bubulcus ibis*	1551	KJ190945
Pelecaniformes	*Butorides striata*	1551	KJ190953
Pelecaniformes	*Dupetor flavicollis*	1551	KJ190957
Pelecaniformes	*Egretta eulophotes*	1551	KJ190949
Pelecaniformes	*Egretta garzetta*	1551	KJ190950

Pelecaniformes	*Egretta sacra*	1551	KJ190951
Pelecaniformes	*Eudocimus ruber*	1551	KR862292
Pelecaniformes	*Gorsachius goisagi*	1551	KT364530
Pelecaniformes	*Gorsachius magnificus*	1551	KT364529
Pelecaniformes	*Gorsachius melanolophus*	1551	KT364531
Pelecaniformes	*Ixobrychus cinnamomeus*	1551	KJ190959
Pelecaniformes	*Ixobrychus eurhythmus*	1551	KJ190956
Pelecaniformes	*Ixobrychus sinensis*	1551	KJ190958
Pelecaniformes	*Leucocarbo chalconotus*	1551	GU071054
Pelecaniformes	*Morus serrator*	1551	GU071056
Pelecaniformes	*Nannopterum brasilianus*	1551	KT626611
Pelecaniformes	*Nipponia nippon*	1551	MN047457
Pelecaniformes	*Nycticorax nycticorax*	1551	KJ190954
Pelecaniformes	*Phaethon lepturus*	1551	KR349465
Pelecaniformes	*Phaethon rubricauda*	1551	AP009043
Pelecaniformes	*Phalacrocorax carbo*	1551	KR215630
Pelecaniformes	*Platalea leucorodia*	1551	KT901459
Pelecaniformes	*Platalea minor*	1551	EF455490
Pelecaniformes	*Threskiornis aethiopicus*	1551	GQ358927
Phoenicopteriformes	*Phoenicopterus roseus*	1551	EF532932
Phoenicopteriformes	*Phoenicopterus ruber*	1551	KT159835
Piciformes	*Campephilus guatemalensis*	1551	KT443920
Piciformes	*Campephilus imperialis*	1551	KU158198
Piciformes	*Dendrocopos canicapillus*	1551	MK335534
Piciformes	*Dendrocopos darjellensis*	1551	MK335535
Piciformes	*Dendrocopos leucotos*	1551	KU131555
Piciformes	*Dendrocopos major*	1551	KT350609
Piciformes	*Dryocopus pileatus*	1551	DQ780879
Piciformes	*Indicator maculatus*	1551	MK060132

Piciformes	*Indicator xanthonotus*	1551	MH737741
Piciformes	*Jynx ruficollis*	1551	MK060133
Piciformes	*Megalaima virens*	1557	MK060136
Piciformes	*Picoides pubescens*	1551	KT119343
Piciformes	*Picumnus innominatus*	1551	KX831678
Piciformes	*Picus canus*	1551	MK348064
Piciformes	*Prodotiscus insignis*	1551	MK060143
Piciformes	*Pteroglossus azara flavirostris*	1551	DQ780882
Piciformes	*Sasia ochracea*	1551	KT443919
Podicipediformes	*Podiceps cristatus*	1551	AP009194
Podicipediformes	*Tachybaptus novaehollandiae*	1551	EF532936
Podicipediformes	*Tachybaptus ruficollis*	1551	KJ913674
Procellariiformes	*Aphrodroma brevirostris*	1551	AY158678
Procellariiformes	*Daption capense*	1551	MH924023
Procellariiformes	*Hydrobates castro*	1551	MH433599
Procellariiformes	*Phoebastria albatrus*	1551	KJ735514
Procellariiformes	*Phoebastria immutabilis*	1551	KJ735513
Procellariiformes	*Phoebastria nigripes*	1551	KJ735512
Procellariiformes	*Procellaria cinerea*	1551	AP009191
Procellariiformes	*Thalassarche chrysostoma*	1551	AP009193
Procellariiformes	*Thalassarche melanophrys*	1551	AY158677
Psittaciformes	*Agapornis lilianae*	1548	MN481406
Psittaciformes	*Agapornis nigrigenis*	1548	MN481405
Psittaciformes	*Agapornis personatus*	1548	MN481404
Psittaciformes	*Agapornis roseicollis*	1548	EU410486
Psittaciformes	*Amazona aestiva*	1548	KT361659
Psittaciformes	*Amazona barbadensis barbadensis*	1548	JX524615
Psittaciformes	*Amazona ochrocephala*	1548	KM611467
Psittaciformes	*Amazona ventralis*	1548	KX925977

Psittaciformes	*Ara ararauna*	1548	KF010315
Psittaciformes	*Ara chloropterus*	1548	MN604694
Psittaciformes	*Ara glaucogularis*	1548	JQ782215
Psittaciformes	*Ara macao*	1548	MK351783
Psittaciformes	*Ara militaris*	1548	KM611466
Psittaciformes	*Ara militaris mexicanus*	1548	JX524613
Psittaciformes	*Ara severus*	1548	KF946546
Psittaciformes	*Ara tricolor*	1548	MG432917
Psittaciformes	*Aratinga nenday*	1548	MK965540
Psittaciformes	*Aratinga solstitialis*	1548	MK343132
Psittaciformes	*Brotogeris cyanoptera*	1548	HM627323
Psittaciformes	*Cacatua moluccensis*	1548	MH133972
Psittaciformes	*Cacatua pastinator*	1548	MH133973
Psittaciformes	*Calyptorhynchus baudinii*	1548	MH133969
Psittaciformes	*Calyptorhynchus lathami*	1548	JF414241
Psittaciformes	*Calyptorhynchus latirostris*	1548	JF414243
Psittaciformes	*Coracopsis vasa*	1554	KM611468
Psittaciformes	*Eclectus roratus*	1548	KM611469
Psittaciformes	*Eolophus roseicapillus*	1548	MH133971
Psittaciformes	*Eupsittula pertinax chrysogenys*	1548	HM640208
Psittaciformes	*Forpus passerinus*	1548	KM611470
Psittaciformes	*Guaruba guarouba*	1548	JQ782217
Psittaciformes	*Lorius chlorocercus*	1548	MN515396
Psittaciformes	*Melopsittacus undulatus*	1548	EF450826
Psittaciformes	*Myiopsitta monachus*	1548	KM611471
Psittaciformes	*Neophema chrysogaster*	1548	JX133087
Psittaciformes	*Nestor notabilis*	1548	MH133967
Psittaciformes	*Nymphicus hollandicus*	1548	MH133968
Psittaciformes	*Orthopsittaca manilatus*	1548	KJ579139

Psittaciformes	*Pionites leucogaster*	1548	MK759905
Psittaciformes	*Pionus chalcopterus chalcopterus*	1548	MF784450
Psittaciformes	*Pionus menstruus menstruus*	1548	KX925978
Psittaciformes	*Poicephalus gulielmi gulielmi*	1548	MF977813
Psittaciformes	*Poicephalus senegalus*	1548	MK749396
Psittaciformes	*Primolius couloni*	1548	KF836419
Psittaciformes	*Primolius maracana*	1548	KJ562357
Psittaciformes	*Prioniturus luconensis*	1548	KM611473
Psittaciformes	*Probosciger aterrimus goliath*	1548	MH133970
Psittaciformes	*Psephotellus pulcherrimus*	1548	KU158195
Psittaciformes	*Psittacara acuticaudatus acuticaudatus*	1548	JQ782214
Psittaciformes	*Psittacara brevipes*	1548	KC936100
Psittaciformes	*Psittacara leucophthalmus*	1548	KF444466
Psittaciformes	*Psittacara mitratus mitratus*	1548	JX215256
Psittaciformes	*Psittacara rubritorquis*	1548	JX524614
Psittaciformes	*Psittacula alexandri*	1548	MK986660
Psittaciformes	*Psittacula derbiana*	1548	MK343133
Psittaciformes	*Psittacula eupatria*	1548	MK343134
Psittaciformes	*Psittacula roseata*	1548	MK986661
Psittaciformes	*Psittacus erithacus*	1548	KM611474
Psittaciformes	*Psittrichas fulgidus*	1548	KM611475
Psittaciformes	*Pyrrhura rupicola*	1548	KF751801
Psittaciformes	*Rhynchopsitta terrisi*	1548	KF010318
Psittaciformes	*Trichoglossus rubritorquis*	1548	MN182499
Rheiformes	*Pterocnemia pennata*	1551	AF338709
Rheiformes	*Rhea americana*	1551	AF090339
Sphenisciformes	*Aptenodytes forsteri*	1551	KT159230
Sphenisciformes	*Aptenodytes patagonicus*	1551	MK801135

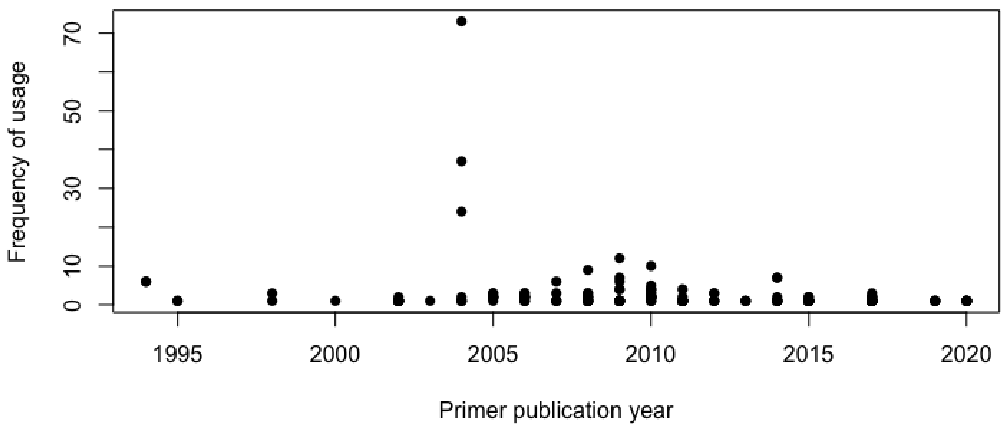
Sphenisciformes	*Eudyptes chrysocome*	1551	AP009189
Sphenisciformes	*Eudyptula minor*	1551	AF362763
Sphenisciformes	*Pygoscelis adeliae*	1551	KC875855
Sphenisciformes	*Pygoscelis antarcticus*	1551	KU356673
Sphenisciformes	*Pygoscelis papua*	1551	KU356677
Sphenisciformes	*Spheniscus demersus*	1551	KC914350
Sphenisciformes	*Spheniscus humboldti*	1551	KM891593
Sphenisciformes	*Spheniscus magellanicus*	1551	KU361806
Sphenisciformes	*Spheniscus mendiculus*	1551	KU361807
Strigiformes	*Asio flammeus*	1551	KP889214
Strigiformes	*Asio otus*	1551	MG916810
Strigiformes	*Bubo bubo*	1551	MK656285
Strigiformes	*Bubo scandiacus*	1551	MG681084
Strigiformes	*Glaucidium brodiei brodiei*	1551	KP684122
Strigiformes	*Glaucidium cuculoides*	1551	KY092431
Strigiformes	*Ninox novaeseelandiae*	1551	AY309457
Strigiformes	*Ninox scutulata*	1551	KT943750
Strigiformes	*Ninox strenua*	1551	KX529654
Strigiformes	*Otus bakkamoena*	1551	KT340631
Strigiformes	*Otus scops*	1551	KT340630
Strigiformes	*Otus sunia*	1551	MF346692
Strigiformes	*Phodilus badius*	1551	KF961183
Strigiformes	*Strix leptogrammica*	1551	KC953095
Strigiformes	*Strix occidentalis caurina*	1551	MF431746
Strigiformes	*Strix uralensis*	1551	MG681082
Strigiformes	*Strix varia varia*	1551	MF431745
Struthioniformes	*Struthio camelus*	1551	AF338715
Tinamiformes	*Eudromia elegans*	1551	AF338710
Tinamiformes	*Tinamus guttatus*	1551	KR149454

Tinamiformes	*Tinamus major*	1551	AF338707
Trogoniformes	*Trogon viridis*	1551	EU410490
Upupiformes	*Upupa epops*	1551	KT356220

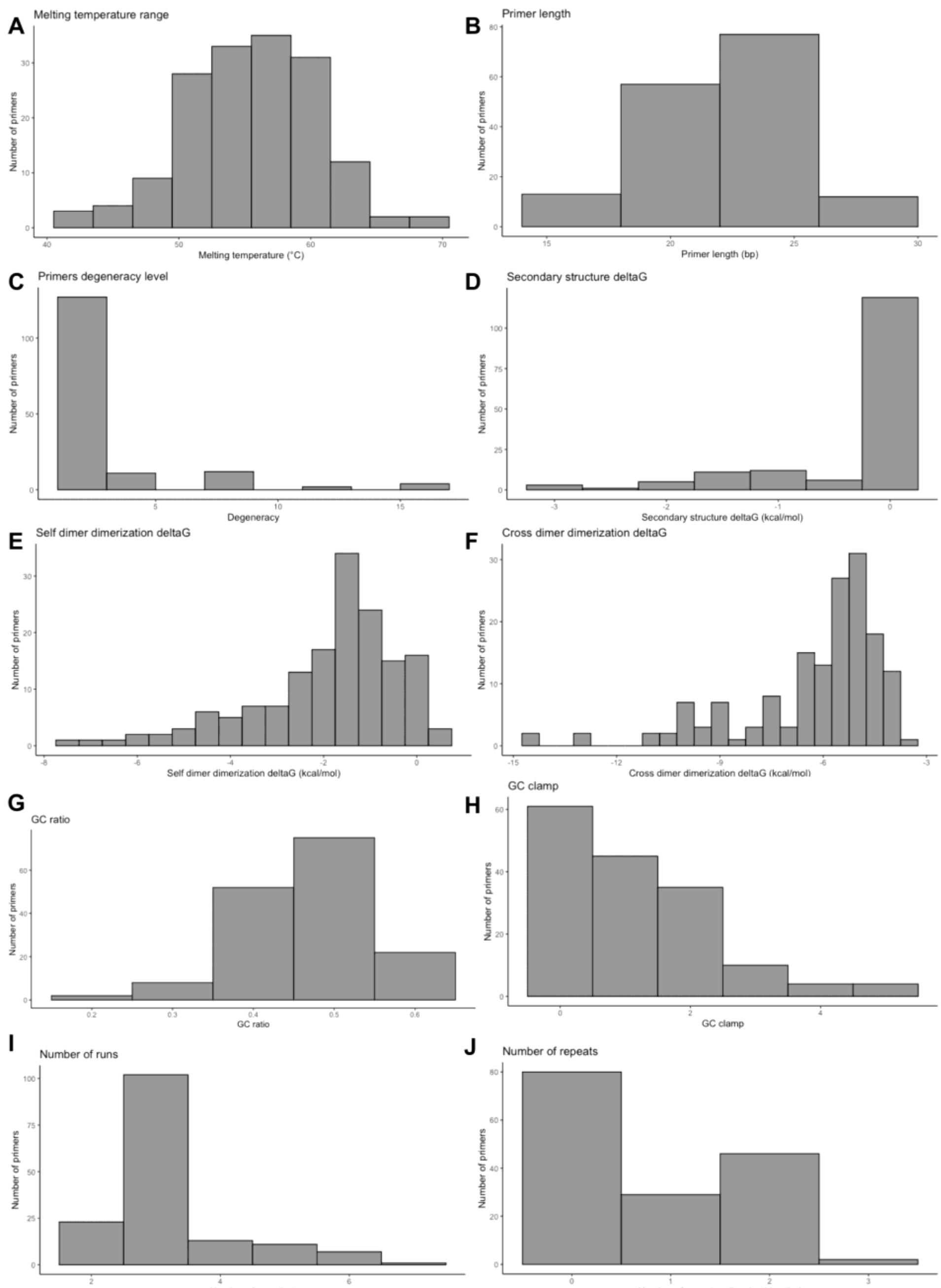
Supplementary Table 4. Number of template sequences covered by each primer that presented at least one binding event on this in silico COI coverage evaluation analysis. Number of template sequences covered by each primer per bird order and as a total per primer.

Link for accessing this supplementary material:
https://drive.google.com/drive/folders/1u_rLK2C-vdP1zVPPpplh3IvrOdN7LofT?usp=sharing

Supplementary Fig. 1 Usage frequency of primers for the COI gene used on birds DNA barcoding studies and its year of publication. A significant negative relation between the primers frequency of usage and its year of publication was observed (r= -0.17, p = 0.01).



Supplementary Fig. 2 Distribution of the evaluated coverage and input constraints as well as the PCR physicochemical properties range of the evaluated COI primer sets available for the avian group. A) Distribution of the primers' observed melting temperature (ºC); B) Distribution of the primers' length (in bp); C) Level of degeneracy observed on the retrieved primers; D) Distribution of the values of free energy available (ΔG) for the formation of primer secondary structures (kcal/mol); E) Distribution of the values of free energy available (ΔG) for the interaction of a primer with itself (kcal/mol); F) Distribution of the values of free energy available (ΔG) for the interaction of a primer with another primer (kcal/mol); G) Distribution of the primers' GC ratio in terms of number in the interval [0,1]; H) Distribution of the GC at the 3' end (GC clamp) observed on the evaluated primers; I) Distribution of the length of homopolymer runs in the evaluated primers (Number of runs – in bp); J) Distribution of the length of dinucleotide repeats in the evaluated primers (Number of repeats – in bp).



Conclusões gerais

Com o presente trabalho, foi possível visualizar o status dos *primers* universais utilizados na identificação de aves utilizando o gene COI, o que auxilia na seleção da combinação de *primers* mais eficientes para níveis de grupos específicos ou mais abrangentes desta classe, de forma a otimizar tempo e recurso financeiro na identificação molecular de espécies. Além disso, foi possível visualizar o atual status da disponibilidade de sequências genômicas para o grupo das aves, mostrando os grupos com maior ou menor disponibilidade dessas sequências e mostrando que ainda faltam muitas espécies com informação genômica a serem disponibilizadas e exploradas em estudos como este.

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