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A non-invasive molecular genetic technique for sex identification of Nicobar pigeon (*Caloenas nicobarica*), the last living relative of Dodo bird

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Abstract Nicobar pigeon (*Caloenas nicobarica*) is the last surviving member of the genus *Caloenas* and the closest living relative of the extinct Dodo bird. It is classified as a near-threatened species that is illegally trapped for consumption, extraction of decorative gizzard stones, and as pets due to its size and attractive appearance. Captive breeding is essential for conservation effort and potential future commercialization of Nicobar pigeon. However, it is difficult due to the sexually-monomorphic and monogamous nature of the species. A non-invasive PCR-based technique for sex identification of Nicobar pigeon was presented here, which provided a fast and reliable method that achieves accurate results within 8 hours. Using feather samples individually obtained from captive Nicobar pigeons, results were also compared between molecular sexing method against traditional morphology-based sexing methods by vent and hackle length. However, no correlation was found between the molecular and morphological methods. Additionally, bioinformatic analysis of the amplified DNA sequence was performed for Nicobar pigeon, which showed its phylogenetic relationship with other members of the pigeon and dove family (Columbidae). This research finding is the first to show a safe, fast, and reliable method for sex identification of *Caloenas* bird. The proposed molecular sexing method is suitable for utilization in captive breeding program, applicable for field deployment, and potentially useful for agricultural applications.

Keywords: Monomorphic bird, PCR, CHD gene

Introduction

Nicobar pigeon (*Caloenas nicobarica*) is a ground-dwelling bird in the pigeon and dove family (Columbidae), Columbiformes order (Gray, 1840). They are medium-sized birds with overall compact appearance including short wings and tail that enable them to fly short distances. Their habitat ranges from the Andaman islands and Malayan archipelago to the islands of South East Asia, although they have become increasingly difficult to find in the wild

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(BirdLife International, 2016). It is due to their attractive appearance including the deep blue-green feather color and elaborately long hackle, which makes them valuable targets for trapping for illegal trades as pets. Additionally, due to the limited flight capability, they are also hunted for meat and gizzard stones. Nicobar pigeon is currently classified as a near-threated (NT) species according to the International Union for the Conservation of Nature (IUCN) Red List. It is protected under Wild Animal Reservation and Protection Act of Thailand, B.E. 2535, due to the loss of habitat and shrinking population size.

Genetic data indicates that Nicobar pigeon is the last surviving relative of the Dodo bird (*Raphus cucullatu*s), which has been believed to be extinct for over 300 years, and currently the last surviving member of the *Caloenas* genus (Heupink *et al.*, 2014; Shapiro *et al*., 2002). Columbiformes (pigeon and dove) lineage originated during the late Cretaceous period and underwent a massive adaptive radiation during the late Oligocene period around 25 million years ago. It has been estimated that the *Caloenas*, including Nicobar pigeon and the extinct spotted green pigeon (*Caloenas maculata*), diverged from its extinct sister lineage that includes the Dodo bird and Rodrigues solitaire (*Pezophaps solitaria*) about 18-20 million years ago (Soares *et al*., 2016). This makes the conservation of Nicobar pigeon an extremely important and urgent matter to protect both the biodiversity and its genetic resources.

Captive breeding of Nicobar pigeon has been difficult due to their sexually monomorphic appearance and monogamous mating behavior. Male and female birds are virtually non-distinguishable morphologically and behaviorally. Moreover, females typically only lay 1-2 eggs per clutch, and fertilized eggs need approximately 21-24 days of incubation before hatching. Therefore, the reproduction rate of Nicobar pigeon is much slower than the rate at which they are trapped and hunted. The relative rarity and breeding difficulty of Nicobar pigeon makes it necessary to establish a non-invasive, accurate and reliable technique to identify male and female birds to facilitate breeding in captivity, such as in zoos and wildlife breeding facilities. Currently, existing approaches for bird sexing include 1) vent sexing by inspecting the morphology of the cloaca and width of the pelvic girdle, which requires specific training of personnel and handing of the animal (Boersma and Davies, 1987; Jackson, 2006; Totterman, 2015), 2) karyotyping by microscopic examination of sex chromosomes within the blood cells, which is relatively slow and requires specialized equipment (Makino *et al.*, 1956) and 3) molecular sexing by polymerase chain reaction (PCR) (Çakmak *et al.*, 2017), which is currently the most reliable and robust assay that can be done on virtually all sample types that contains nucleic acid such as blood, tissue, and feather, including degraded samples in long-term storage (Horváth *et al*., 2005).

Traditionally, molecular sexing of avian species can be done by distinguishing the size of the genomic region within the sex-linked *CHD1* (chromodomain helicase DNA binding 1) gene, which is presented in two copies, each with different intronic lengths, a shorter one residing on the W chromosome and a longer one on the Z chromosome (Ellegren, 1996; Griffths *et al.*, 1996). Because male birds possess homogametic sex chromosome (ZZ) and female birds possess heterogametic sex chromosome (ZW), PCR amplification of the intronic region of *CHD1* gene yields one long product (single band) in male birds and two products (double band) with different sizes in female birds.

To date, PCR-based assays for molecular sexing has been successfully performed in a wide range of avian species with the exception of ratite birds such as ostrich and emu (Çakmak *et al.*, 2017; Cerit and Avanus, 2007; St. John and Quinn, 1998). Notably, PCR sexing has been done in endangered monomorphic bird species such as cranes (Liu *et al*., 2011) and Ara species (Bermúdez-humarán *et al*., 2002), which provide valuable information for conservation and propagation of the species. However, despite extensive genetic and genomic studies in Columbiformes (Holt *et al.*, 2017; Shapiro *et al*., 2013; Stringham *et al.*, 2012), to our knowledge, molecular sexing of *Caloenas* birds including Nicobar pigeon has never been successfully performed.

In this study, the molecular sexing of Nicobar pigeon was performed to develop a non-invasive, rapid, and reliable PCR-based method that can be completed within 8 hours and required minimal equipment and reagents that should be readily available in most laboratories. Additionally, Nicobar pigeon's bioinformatic and phylogenetic analysis with other bird species were performed using the amplified partial sequence of Nicobar pigeon *CHD1* gene.

Materials and methods

Sample collection

Feathers were collected with permission from 18 captive Nicobar pigeons (*Caloenas nicobarica*) which were kept in an aviary at Huai Sai Wildlife Breeding Center, Cha-am district, Phetchaburi province, Thailand (Figure 1). Briefly, birds were held by an animal handler, and the feathers were gently pulled from the distal part of the wing area and stored in individually-labeled plastic bags kept at room temperature. Each bird was individually tracked using a leg band according to the standard protocol of Thailand Department of National Parks, Wildlife, and Plant Conservation. Birds were

released back into the aviary and monitored for at least 1 hour to ensure safe return to the flock and display of normal behavior. During feather collection, morphological data was also collected and recorded for each individual bird. Hackle length was measured using a small tape measure from the base to the tip of the longest neck feather, and cloaca width (between pelvic girdle) was measured using a vernier scale. All animal procedure was done in accordance with the protocol reviewed and approved by Silpakorn University Animal Care and Use Committee (Project ID: 02/2562).

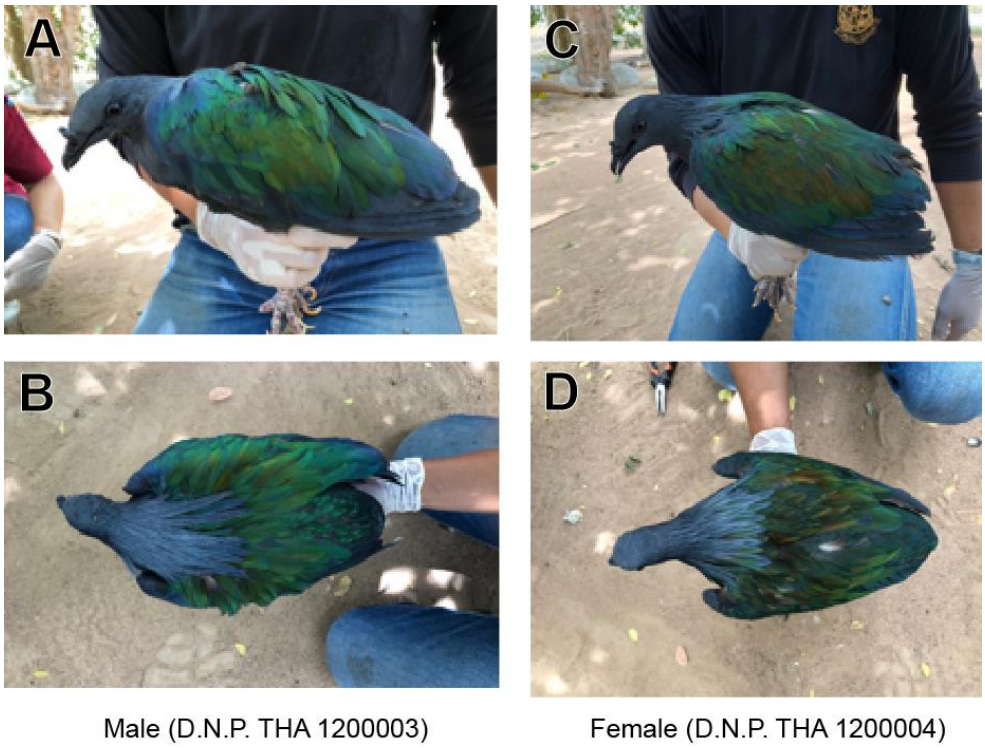

Figure 1. Representative images of male (A and B) and female (C and D) Nicobar pigeons (*Caloenas nicobarica*). Birds were imaged and tagged with leg bands for identification. Feathers were collected from wing region and used for DNA extraction and sexing by PCR. Note that the birds were sexually monomorphic and are virtually indistinguishable in physical appearance

DNA extraction

DNA was extracted from the feather samples using a modified protocol (De Volo *et al.*, 2008) (Figure 2). Briefly, feather was cut at the base below the superior umbilicus, rinsed briefly once with ethanol and twice with sterile

distilled water in a petri dish, then cut into small pieces (2-3mm) and transferred to a 2 ml tube. Feather pieces were digested in Digestion Mix solution [600µl of 1x TNE (100mM NaCl, 50mM Tris-HCl, 25mM EDTA, pH 7.5), 60µl of 1M Tris-HCl, 1µl of proteinase K (20mg/ml), 10µl of 25% SDS, and 80µl of 1M DTT] at 55℃ for 1 hour or until completely dissolved. Proteins were precipitated by adding 233µl of 7.5M ammonium acetate, vortex briefly, frozen at -20℃ for 20 minutes, and centrifuged at 13,000rpm at 5℃ for 10 minutes. Supernatant was transferred to a new tube, mixed thoroughly with 600µl of isopropanol and 1µl of glycogen, frozen at -20℃ for 1 hour, and centrifuged at 13,000rpm at 5℃ for 15 minutes. Supernatant was discarded and DNA pellet was washed briefly with 600µl of 70% ethanol, then centrifuged again at 13,000rpm at 5℃ for 2 minutes. Ethanol was discarded and DNA pellet was air dried at room temperature. Pellets were resuspended in 50µl sterile distilled water and DNA quantified using a spectrophotometer.

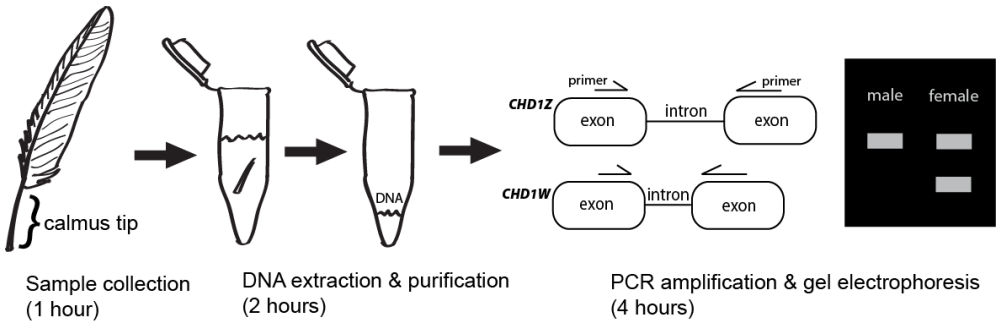

Figure 2. Summary of the workflow

PCR and sequencing

PCR was performed using 1µl of the extracted DNA and 24µl of 1x OneTaq PCR master mix (NEB, USA), which contains a blend of Taq and high fidelity Deep Vent. Primers used were: CHD1F (5'-TATCGTCAGTTTCCTTTTCAGGT-3'), CHD1R (5'-CCTTTTATTGATCCATCAAGCCT-3'), 2550F (5'-GTTACTGATTCGTCTACGAGA-3'), and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') (Çakmak *et al*., 2017). Thermal cycling condition is as follow: pre-denaturation at 94℃ for 30 seconds, denaturation at 94℃ for 30 seconds, annealing for 30 seconds at decreasing temperature from 57℃ to 50℃ (decreasing 1℃ each cycle) for 6 cycles, extension at 68℃, followed by 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 50℃ for 30 seconds, and extension at 68℃ for 30 seconds. A touchdown

protocol was used to ensure primer binding specificity during the annealing step. PCR products were analyzed by running on a 1% agarose gel electrophoresis in 0.5% TBE buffer at 100 volt for 20 minutes and visualized under gel documentation equipment. To ensure accurate identification, sample from each bird was tested by PCR at least twice before sex was assigned. The remaining PCR product was purified using GenepHlow gel/PCR purification kit (Geneaid, Taiwan), quantified using a spectrophotometer and sent for sequencing by standard Sanger protocol (U2Bio, Thailand). The time taken to perform DNA extraction, PCR and DNA purification is typically done under 8 hours.

Data analysis

DNA sequence of Nicobar pigeon (*Caloenas nicobarica*) was trimmed for high quality chromatogram peaks and analyzed using BLAST (basic local alignment search tool; https://blast.ncbi.nlm.nih.gov/) against nucleotide collection database (National Center for Biotechnology Information; NCBI). Multiple sequence alignment was performed and phylogenetic tree was constructed by neighbor-joining method, Jukes-Cantor genetic distance mode using Geneious version R9 (Biomatters, USA). The DNA sequences of the species for genetic comparison were the following accession numbers: LR594555, JF279562, AY517719, GU451226, GU451225, MF662599, GU451227, GU451228, EU814908.

Results

Molecular sexing of Nicobar pigeon

Feather samples were collected from a total of 18 captive Nicobar pigeons from Huai Sai Wildlife Breeding Center, Cha-am district, Phetchaburi province, Thailand, without prior knowledge of the birds' age or sex. Initially, both pairs of CHD1F/CHD1R and 2550F/2718R primers were used to amplify the intronic region of *CHD1* gene. However, after optimization and implementation of a touchdown PCR protocol, only the CHD1F/CHD1R primer pair was able to amplify the region. Running the PCR product on agarose gel electrophoresis revealed the expected single band around 550bp for males (n=6) and two bands at approximately 300 and 550bp for females (n=12) (Figure 3). Therefore, the subsequent PCR rounds were performed using CHD1F/CHD1R primer pair. All 18 birds were successfully sex-identified using this PCR method; six were males and twelve were females (Table 1).

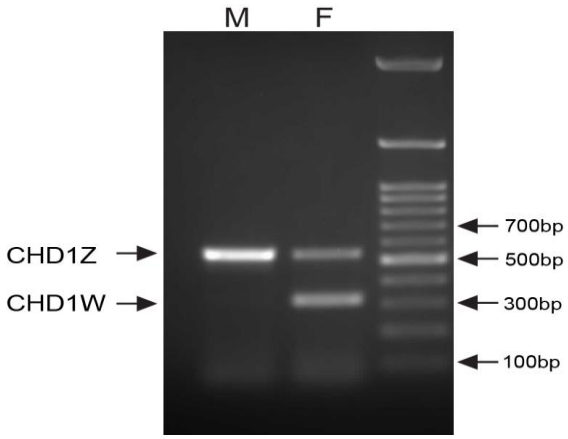

Figure 3. Representative results of Nicobar pigeon (*Caloenas nicobarica*) sexing by PCR and agarose gel electrophoresis. PCR product was amplified with CHD1F and CHD1R primers, using a touchdown protocol, and resolved by running on a 1% agarose gel electrophoresis (see text for details). Lane 1: male, lane 2: female, lane 3: DNA ladder standard (selected band sizes indicated by arrows on the right)

Table 1. Morphological characterization using hackle length and cloaca width, and PCR sexing results of each of the 18 Nicobar pigeons (*Caloenas nicobarica*)

Leg band ID	Hackle length (cm)	Cloaca width (cm)	PCR sexing
D.N.P. THA 1200001	8.0	1.0	male
D.N.P. THA 1200002	5.0	2.0	female
D.N.P. THA 1200003	9.0	2.2	male
D.N.P. THA 1200004	8.0	1.1	female
D.N.P. THA 1200005	6.5	0.9	female
D.N.P. THA 1200006	8.0	1.1	female
D.N.P. THA 1200007	7.0	0.5	female
D.N.P. THA 1200008	8.0	0.3	female
D.N.P. THA 1200009	7.0	1.1	female
D.N.P. THA 1200010	8.2	2.2	male
D.N.P. THA 1200011	6.0	1.0	female
D.N.P. THA 1200012	6.0	1.1	female
D.N.P. THA 1200013	6.0	0.2	male
D.N.P. THA 1200014	9.0	1.3	male
D.N.P. THA 1200015	7.5	1.9	female
D.N.P. THA 1200016	6.0	0.2	female
D.N.P. THA 1200017	7.0	0.1	female
D.N.P. THA 1200018	6.0	1.7	male

Morphological data comparison

In addition to sexing by molecular method, the morphological data from each of the Nicobar pigeons were collected for comparison. Out of the 18 Nicobar pigeons sexed by PCR, hackle length and cloaca width were individually measured and recorded (Table 1). Males and females showed average hackle lengths of 7.7cm and 6.8cm, respectively, and the length difference between sexes was not statistically significant ($p=0.14$, two-tailed t-test). Similarly, the average cloaca widths of the males and females were 1.4cm and 0.9cm, respectively, which were also not significantly different ($p=0.23$, two-tailed t-test) (Table 2). Therefore, the hackle length and cloaca width were not sexually dimorphic and may not be good indicators of sex in Nicobar pigeon.

Table 2. Hackle length and cloaca width of the 18 Nicobar pigeons (*Caloenas nicobarica*), which were molecular sexed by PCR, showed no statistically significant difference between male and female birds. Numbers are average ± standard deviation

PCR sexing	Hackle length (cm)	Cloaca width (cm)
Male	7.70 ±1.38	1.43 ±0.77
Female	6.83 ±0.96	0.94 ±0.60

DNA sequence alignment and phylogenetic analysis

The amplified partial sequence of the Nicobar pigeon *CHD1* gene from a representative male bird was performed DNA sequencing and bioinformatic analysis. The result showed 509 bp quality-trimmed read which was used for nucleotide BLAST against NCBI nucleotide collection database. Top 3 BLAST hits were all from the Columbidae family: European turtle dove (*Streptopelia turtur*), common wood pigeon (*Columba palumbus*), and rock dove (*Columba livia*). The percentage of pairwise identical sequence was between 89-93%, and bit score of 596-732. Aligning the Nicobar pigeon sequence with the 3 top BLAST hits revealed that the *CHD1* gene region was highly similar between the 4 species, with *Streptopelia turtur* being the closest in sequence similarity (Figure 4). Nicobar pigeon possesses unique mutations which diverged from the rest of the pigeons and doves, such as a TCC→GAT mutation at position 90-92, a unique 2bp deletion at position 265-266, 411-412, 427-428, and single nucleotide substitutions at position 97, 126, 146, 172, 174, 188, 333, 340, 347, 355, 358, 391, 420, 456, and 469.

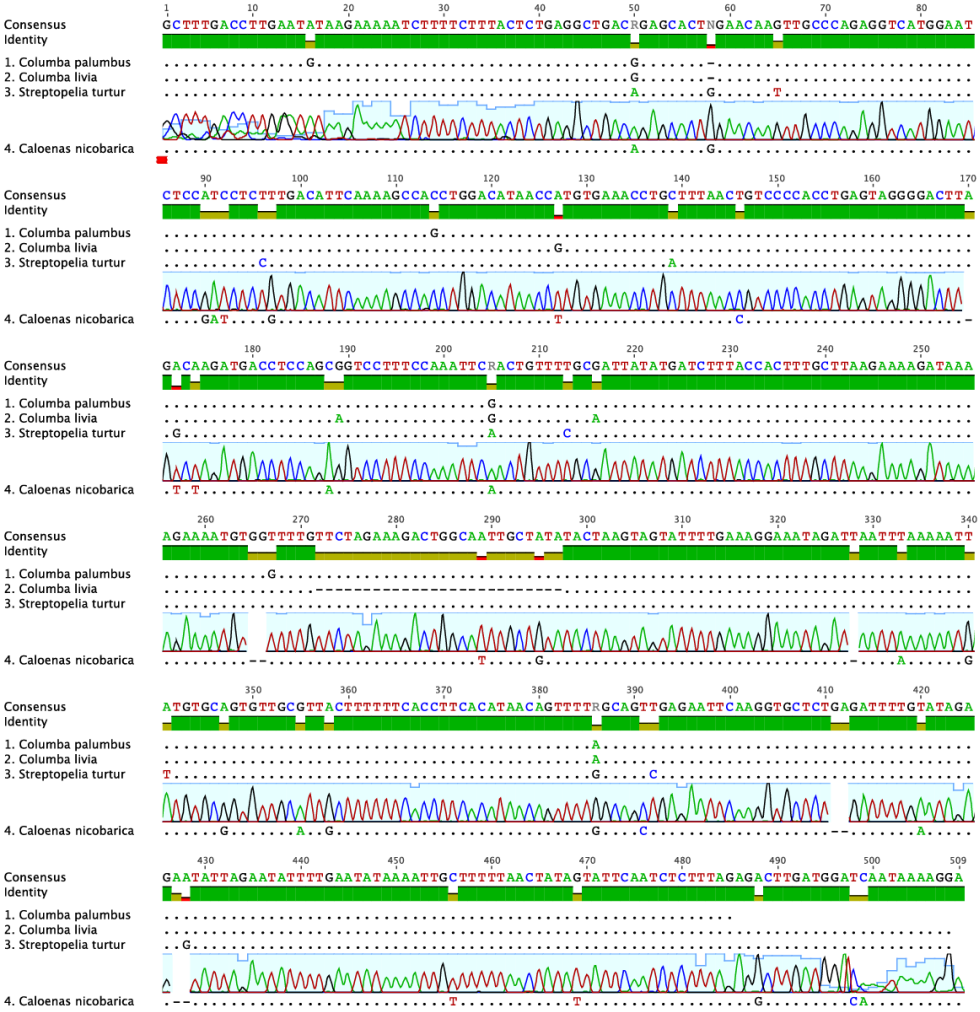

Figure 4. Sequence alignment of Nicobar pigeon (*Caloenas nicobarica*) partial *CHD1* gene and related birds within the dove and pigeon family (Columbidae) from top BLAST results (*Columba palumbus*, *Columba livia*, *Streptopelia turtur*). A dot (.) indicates an identical sequence, and a dash (-) indicates a deletion. Sequence alignment identity is shown by the bar height (green, yellow, red) below the consensus sequence. Chromatogram shows the sequence of a representative male Nicobar pigeon

Phylogenetic relationship between Nicobar pigeon and other related bird species was shown by constructing a phylogenetic tree from a sequence alignment of Nicobar pigeon *CHD1* partial gene sequence and the sequences from top BLAST hits with bit-score 430 and above (E-value between 0 and 3.64e-118) using neighbor-joining tree building method and Jukes-Cantor

genetic distance model. Nicobar pigeon sequence appeared as a cluster with other pigeons and doves of the Columbidae family, and remained further distant away from other non-Columbidae bird species (Figure 5). However, the sequence of Nicobar pigeon was still distinctly unique and diverged early from the rest of the Columbidae family.

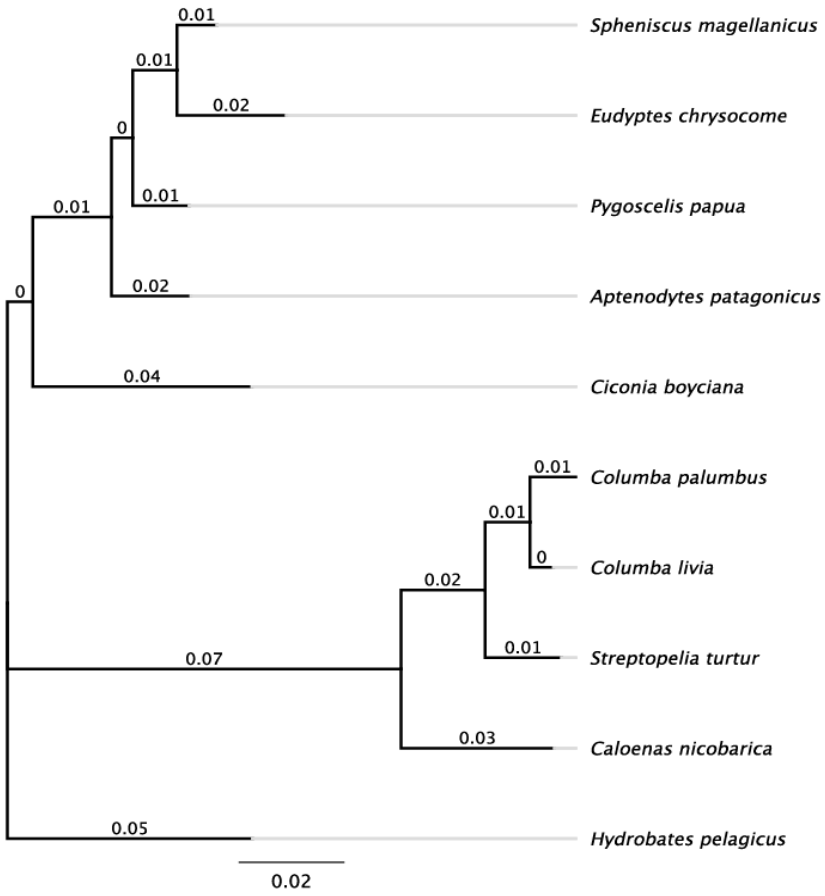

Figure 5. Phylogenetic tree shows relationship between Nicobar pigeon (*Caloenas nicobarica*) and related bird species. Tree was constructed from sequence alignment of Nicobar pigeon *CHD1* gene (partial sequence) and sequences from BLAST hits with bit-score 430 and above, E-value between 0 and 3.64e-118, Genbank accession number (LR594555 JF279562 AY517719 GU451226 GU451225 MF662599 GU451227 GU451228 EU814908), using Neighbor-joining tree building method and Jukes-Cantor genetic distance model. Branch length indicates number of nucleotide substitutions per site

Discussion

The results of this study showed an effective molecular method to identify Nicobar pigeon sex unambiguously, rapidly, and safely using feather samples, which can be applied for use in captive breeding programs. All Nicobar pigeon samples were amplified by PCR, and sex of birds were quickly and unambiguously assigned. Previous study showed that two primer sets were able to amplify the partial *CHD1* gene across multiple lineages of bird species (Çakmak *et al.*, 2017). However, in this research finding, only one pair of primers, CHD1F/CHD1R, successfully amplified the sexually dimorphic region of Nicobar pigeon *CHD1* gene. The difference in amplification efficiency was likely due to the sequence differences within the primer binding regions.

In monomorphic bird species, where physical appearance between males and females are similar, small but measurable morphological differences may still exist. For example, hackle length is generally longer in male birds (Cuervo and Moller, 2000; Kessel, 1951), and cloaca width is wider in females (Boersma and Davies, 1987; Jackson, 2006; Totterman, 2015). However, results of this study showed no statistically significant difference between male and female Nicobar pigeons. This is likely because the hackle length can be longer in older birds regardless of sex (Kessel, 1951), and the cloaca width can expand and contract in female birds before and after laying eggs (Boersma and Davies, 1987; Jackson, 2006). In conclusion, the morphological measurements of hackle and vent are currently not a reliable method to determine Nicobar pigeon sex. However, in the future, increasing the sample size or careful examination of other anatomical or behavioral traits may reveal other detectable sexual dimorphisms, although this is beyond the scope of the current study.

The *CHD1* gene is highly conserved with slow evolutionary rate in avian species, making it a reliable marker for population and phylogenetic analysis (Ciorpac *et al.*, 2016). Using the amplified DNA sequence from Nicobar pigeon's *CHD1* gene, we performed bioinformatic analysis to examine its evolutionary relationship among bird species. Based on the *CHD1* gene sequence alignment, we found that Nicobar pigeon is closely related to other members of the dove and pigeon family (Columbidae), although several distinct mutations were unique to Nicobar pigeon. Because Nicobar pigeon is the closest surviving relative of the extinct Dodo bird (*Raphus cuculatus*), it is possible that these unique features are also shared with the Dodo bird and others in the genus *Caloenas*, including the extinct spotted green pigeon (*Caloenas maculata*). However, this is only a speculation, as we cannot perform sequence alignment or database search against other *Caloenas* birds,

becasue they are extinct and their DNA sequences for this genomic region is currently not available.

Our phylogenetic tree constructed from *CHD1* gene sequences of Nicobar pigeon and related avian species showed an obvious clustering of Columbiform birds into a distinct clade. Careful examination of the Columbiform clade revealed that Nicobar pigeon sequence diverged from the rest of the clade relatively early. This may be due to the divergence of Columbiformes into two major clades over 25 millions years ago during the late Oligocene period: the Indo-Pacific clade, including Nicobar pigeon, and the Holoarctic/New World clade including rock dove, European turtle dove, and common wood pigeon (Soares *et al*., 2016). This so-called "Columbiform radiation" puts Nicobar pigeon and Dodo bird within the Indo-Pacific clade, which is genetically distinct from other birds within the Holoarctic/New World clade, and this is reflected in the phylogenetic tree presented in this study. Overall, our phylogenetic tree is consistent with those constructed in previous studies from whole mitochondrial genome (Soares *et al.*, 2016) and *12S* gene sequence (Heupink *et al.*, 2014), indicating that the partial *CHD1* gene indeed provides sufficient sequence information for evolutionary genetic analysis.

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