Utility of DNA barcoding for identification of bird-strike samples from India

Bird strikes usually occur due to the entry of aircraft into the bird layer, i.e. the region up to which birds fly in the sky and are common during landing or take-off[1]. Along with the financial losses, bird strike also reduces the mission capability of the crew, loss of flying hours, permanent damages to the aircraft and importantly, it is always associated with the risk of mortality[2–6]. The strategies to reduce the chances of bird strike involve habitat modifications, auditory and visual deterrents, avian radar system and changes in aircraft flight time and route. To implement these strategies, identification of the species involved in the bird strike is important. However, due to the impact of bird strike, the bodies of the birds are damaged beyond recognition. Usually the remains of the birds after a bird strike only include a smear of blood, feathers or tissues. Species identification based on feather morphology and keratin electrophoresis often fails to identify most of the reported bird-strike cases[2,7–10].

DNA barcoding using mitochondrial *Cytochrome c oxidase I* (*COI*) gene has been routinely used for species identification[11]. This technique depends entirely upon the nucleotide sequences of the *COI* gene and hence species identification is possible from any part of the body, even if it is damaged beyond recognition. Usually the *COI* gene sequence of the sample under question is generated and compared with the *COI* gene sequence database. All over the world large-scale efforts are being made to identify bird-strike samples using DNA barcoding[12–15]. It is estimated that the aviation industry in India lose approximately Rs 30 billion/year due to bird strike[16]. Despite their impact on the Indian aviation industry, no attempts are made to identify bird-strike samples using DNA barcoding. In the present study, we have evaluated the utility of DNA barcoding for species identification of birds involved in bird-strike incidences.

We received 52 bird-strike samples from the Indian Navy from 2011 to 2014. In most of the cases, samples were collected on Whatman FTA cards (unless mentioned otherwise). A small piece of FTA card was utilized for DNA isolation using QIAamp® DNA micro kit (Qiagen, Stanford, CA, USA). Samples for which DNA was not obtained in the first attempt were repeated twice (Table 1). PCR amplification of *COI* gene was done using primers mentioned by Dove *et al.*[12]. The PCR products were sequenced using forward and reverse primers with an automated sequencer (3730 DNA analyser, ABI, Hitachi).

The resulting sequences were analysed and edited using ChromasPro to generate FASTA sequences. These sequences were utilized for species identification using species identification engine at BOLD (http://www.boldsystems.org/) and BLAST search at NCBI. Most of the studies involving DNA barcoding of birds used 98% cut-off for species identification. However, studies have shown that this cut-off failed to discriminate most of the species[17]. Therefore we have used a cut-off of 97%, as suggested by Hebert *et al.*[11]. To perform clustering analysis, first sequence match of the query sequence within and outside 97% cut-off was also downloaded (Table 1) and aligned using BioEdit[18]. The Kimura-2-parameter (K2P) model of base substitution was used for clustering analysis in MEGA6 software[19]. The sequences were submitted to NCBI under accession numbers KP975214 to KP975256.

Out of the 52 samples received, DNA isolation and amplification was successful for 43 samples. We failed to isolate DNA from the remaining nine samples, even after repeated extraction, probably because the quantity of blood on the FTA card was insufficient to yield the DNA. Failure to isolate DNA from the FTA card bearing insufficient blood spot has already been reported by Dove *et al.*[20]. Out of the 43 samples, 40 (93%) could be accurately identified up to the species level (Table 1). In most of the cases, the second closest species match for these samples was outside the 97% cut-off (Table 1). For three samples (NBS/MIG_810, NBS/DO_246 and NBS/RAJ_2011_07), the identification could not be done because the database does not have *COI* gene sequences for these species. Although all over the world large-scale efforts are being made to build DNA barcode databases for birds[21], studies on DNA barcoding of birds from India are limited[22]. Our analysis reveals that the birds involved in the bird strike belong to 16 different species (Table 1). Neighbour joining (NJ) analysis also identified the unknown samples, as they formed cohesive clades with their respective sequence match and not with the other species (Figure 1).

Species such as *Tyto alba* ($n = 6$) *Vanellus indicus* ($n = 6$) and *Apus affinis* ($n = 4$) showed a high number of incidences of bird strike (Table 1). Small water pools and grasses are common sites near the airports, attracting many invertebrates and other animals that are food sources for such birds. Abundant availability of food in the vicinity of the airports could be one of the reasons that they are the most commonly struck species. These species were also reported in many bird-strike cases from different regions of the world[3,12,23,24]. In the present study, along with *T. alba* we also detected another species of raptors, *Milvus migrans* that feed on lizards and small mammals found in such habitats. The presence of such preys in the gut content of bird-strike samples was also shown using the meta-barcoding studies[25].

The occurrence of species other than birds although uncommon, poses problems for air safety. In this study, we also found three bat species, viz. *Chaerephon plicatus* ($n = 2$), *Pteropus lylei* ($n = 2$) and *Rousettus leschenaultii* ($n = 1$) (Table 1). Due to their flying ability at night, presence of bats in the airports could also be threatening. Thus, this study shows that DNA barcoding can accurately identify non-avian bird-strike samples, which otherwise would not have been possible using traditional methods.

Air safety management of bird strike involves bird dispersal management, shelter management, food source management, water habitat management, etc. Correct species identification is important for the success of these practices. DNA barcoding offers a fast and reliable technique for species identification compared to traditional methods because if blood spots or damaged tissues are provided traditional methods very often failed to identify the species.

Table 1. Species identification of bird-strike samples using BLAST tool at NCBI and species identification tool at BOLD. Sequence similarity match is shown in percentage. For species identification 97% cut-off was used

	BLAST				BOLD identification tool			
Sample code	First species match	(%)	Second closet species	(%)	First species match	(%)	Second closet species	(%)
NBS/DO244A	*Vanellus indicus*	100	*Hoplopterus spinosus*	92	*Vanellus indicus*	99.33	*Vanellus spinosus*	91.63
NBS/GAR 2011 02	*Vanellus indicus*	100	*Hoplopterus spinosus*	92	*Vanellus indicus*	99.33	*Vanellus spinosus*	91.63
NBS/DOCG 755	*Vanellus indicus*	100	*Hoplopterus spinosus*	92	*Vanellus indicus*	99.33	*Vanellus spinosus*	91.63
NBS/RAJ 2011 06A	*Vanellus indicus*	100	*Hoplopterus spinosus*	92	*Vanellus indicus*	99.33	*Vanellus spinosus*	91.63
NBS/RAJ 2011 06	*Vanellus indicus*	100	*Hoplopterus spinosus*	92	*Vanellus indicus*	99.33	*Vanellus spinosus*	91.63
NBS/DO246	*Vanellus indicus*	100	*Hoplopterus spinosus*	92	*Vanellus indicus*	99.33	*Vanellus spinosus*	91.63
NBS/HAN 2012 06	*Corvus splendens*	100	*Corvus macrorhynchos*	96	*Corvus splendens*	99.84	*Corvus kubaryi*	95.94
NBS/CH483/SHI 2012 01	*Corvus splendens*	100	*Corvus macrorhynchos*	96	*Corvus splendens*	99.84	*Corvus kubaryi*	95.94
NBS/SHI 2011 2	*Columba livia*	99	*Columba oenas*	93	*Columba livia*	99.82	*Columba rupestris*	97.63
NBS/SHI/2011/01	*Columba livia*	99	*Columba oenas*	93	*Columba livia*	99.82	*Columba rupestris*	97.63
NBS/SHI 2013 01	*Columba livia*	99	*Columba oenas*	93	*Columba livia*	99.82	*Columba rupestris*	97.63
NBS/GAR 2011 01	*Bubulcus ibis*	100	*Ardea alba*	94	*Bubulcus ibis*	100	*Ardea alba*	93.7
NBS/DO 226	*Tyto alba*	99	*Tyto furcata*	94	*Tyto alba*	99.3	*Tyto alba guttata*	93.88
NBS/RAJ 2011 02	*Tyto alba*	99	*Tyto furcata*	94	*Tyto alba*	99.3	*Tyto alba guttata*	93.88
NBS/CH 483	*Tyto alba*	99	*Tyto furcata*	94	*Tyto alba*	99.3	*Tyto alba guttata*	93.88
NBS/DEG 2011 03	*Tyto alba*	99	*Tyto furcata*	94	*Tyto alba*	99.3	*Tyto alba guttata*	93.88
NBS/GAR 2011 04	*Tyto alba*	99	*Tyto furcata*	94	*Tyto alba*	99.3	*Tyto alba guttata*	93.88
NBS/RAJ 2011 03	*Tyto alba*	99	*Tyto furcata*	94	*Tyto alba*	99.3	*Tyto alba guttata*	93.88
NBS/RAJ 2011 04	*Athene brama*	99	*Athene noctua*	88	NA			
NBS/RAJ 2011 07*	NA		*Vanellus cinereus*	94	NA			
NBS/13/RAJ/2011*	NA		*Vanellus cinereus*	94	NA			
NBS/BS2	*Eremopterix griseus*	99	*Mirafra assamica*	90	*Eremopterix griseus*	99.26	*Eremopterix nigriceps*	92.26
NBS/CGDO 778[¥]	*Apus affinis*	99	*Apus apus*	96	*Apus affinis*	99.69	*Apus apus*	96.38
NBS/DEG 2011 02[¥]	*Apus affinis*	99	*Apus apus*	96	*Apus affinis*	99.69	*Apus apus*	96.38
NBS/KAMOV58217_10[¥]	*Apus affinis*	99	*Apus apus*	96	*Apus affinis*	99.69	*Apus apus*	96.38
NBS/HAN2012 05[¥]	*Apus affinis*	99	*Apus apus*	96	*Apus affinis*	99.69	*Apus apus*	96.38
NBS/HAN 2012 07	*Calandrella brachydactyla*	99	*Calandrella cheleensis*	92	*Calandrella brachydactyla*	99.68	*Calandrella acutirostris*	92.3
NBS/DO 245	*Pluvialis fulva*	99	*Pluvialis dominica*	97	*Pluvialis fulva*	100	*Pluvialis dominica*	95.41
NBS/Helicopter 8F12	*Pluvialis fulva*	99	*Pluvialis dominica*	95	*Pluvialis fulva*	100	*Pluvialis dominica*	95.41
NBS/Helicopter	*Ardeola bacchus*	97	*Ardeola ralloides*	94	*Ardeola bacchus*	97.02	*Ardeola ralloides*	94.19
NBS/MIG810*	NA		*Apus pacificus*	93	NA			
NBS/CH429	*Charadrius leschenaultii*	97	*Charadrius sanctaehelenae*	93	*Charadrius leschenaultii*	97.13	*Charadrius mongolus*	96.46
NBS/CH 486	*Charadrius leschenaultii*	97	*Charadrius sanctaehelenae*	93	*Charadrius leschenaultii*	97.13	*Charadrius mongolus*	96.46
NBS/HAN 2011 01	*Charadrius leschenaultii*	97	*Charadrius sanctaehelenae*	93	*Charadrius leschenaultii*	97.13	*Charadrius mongolus*	96.46
NBS/CHET AK 809	*Microcarbo niger*	99	*Phalacrocorax melanoleucos*	92	*Microcarbo niger*	100	*Phalacrocorax melanoleucos*	92.21
NBS/Dornier 237[¥]	*Milvus migrans*	99	*Haliastur sphenurus*	94	*Milvus migrans*	99.68	*Haliastur sphenuris*	93.69
NBS/HAN 2012 02[¥]	*Milvus migrans*	99	*Haliastur sphenurus*	94	*Milvus migrans*	99.68	*Haliastur sphenuris*	93.69
NBS/DEG 2011 01[¥]	*Milvus migrans*	99	*Haliastur sphenurus*	94	*Milvus migrans*	99.68	*Haliastur sphenuris*	93.69
NBS/MIG MB 673[#]	*Chaerephon plicatus*	100	*Chaerephon* sp.	91	*Chaerephon plicatus*	100	*Chaerephon cf. nigeriae*	92.28
NBS/KAMOV 582[#]	*Chaerephon plicatus*	100	*Chaerephon* sp.	91	*Chaerephon plicatus*	100	*Chaerephon cf. nigeriae*	92.28
NBS/SHI 2012 04[#¥]	*Pteropus lylei*	99	*Pteropus dasymallus*	96	*Pteropus lylei*	98.38	*Pteropus dasymallus*	94.57
NBS/SHI 2012 03[#¥]	*Pteropus lylei*	99	*Pteropus dasymallus*	96	*Pteropus lylei*	98.38	*Pteropus dasymallus*	94.57
NBS/MIG29K[#]	*Rousettus leschenaultii*	100	*Rousettus aegyptiacus*	94	*Rousettus leschenaultii*	100	*Rousettus aegyptiacus*	94.01
NBS/RAJ/2011/01[Ø]								
NBS/RAJ/2011/05[Ø]								
NBS/SHRMK 51[Ø]								
NBS/MIGMB 674[Ø]								
NBS/SHI/2012/01[Ø]								
NBS/SHI/2012/02[Ø]								
NBS/DO228[Ø]								
NBS/Kamov 582 30_10_13[Ø]								
NBS/GAR/2011/03[Ø]								

*Samples that are not identified till species level. [#]Samples that are identified as species of bats. [Ø]Samples from which DNA was not obtained. [¥]Sample showing multiple species within 97% cut-off. For example, samples belonging to *Pteropus lylei* also matched with *Pteropus dasymallus* within 97% cut-off. These two species are closely related and due to morphological variations, they are often misidentified[26]. Similarly, *Apus apus* and *Apus nipalensis* are synonymized species (avibase.bsc-eoc.org), and *Charadrius leschenaultii* and *C. mongolous* are morphologically similar species (http://www.environment.gov.au/), which might lead to misidentification of the species.

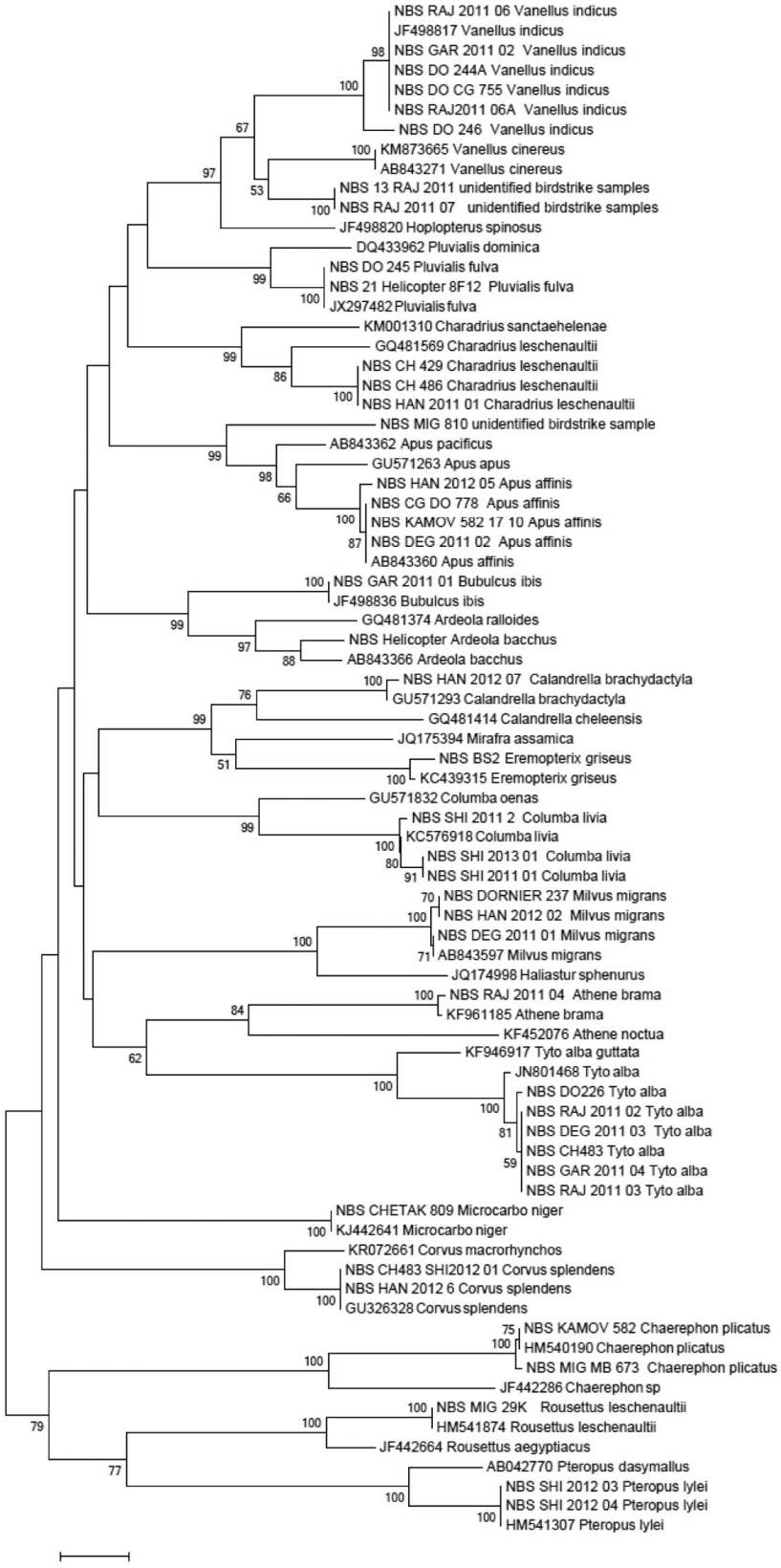

Figure 1. Neighbour joining (NJ) analysis of the bird-strike samples used in this study. The sequences downloaded are shown with their accession numbers. The unknown samples formed cohesive clades with their sequence match downloaded from the database, while the second closest species formed a completely different clade. The unidentified samples do not form such a clade and thus are found on distinct nodes. NJ clustering analysis was performed using MEGA6 software.

A comprehensive study across the country would generate a database that could be used by aviation safety managers to implement better management strategies, lowering the bird-strike incidences and reducing the financial losses as well as loss of human lives.

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Performance of *Apis mellifera* L. in cabbage hybrid seed production in net houses

Cabbage, *Brassica oleracea* var. *capitata* is a highly cross-pollinated species. For the seed production of open pollinated varieties as well as hybrids, an isolation distance of about 1.6 km is recommended[1]. Generally, such isolated distances are difficult to achieve in the field due to the cross compatibility of the crop with other members of cole group, namely cauliflower, knol khol, broccoli, etc. An alternative is to use insect-proof enclosures for seed production.

The pollen of cabbage is heavy and sticky, and hence not easily wind-borne; therefore, it is dependent upon various pollinators for pollination. A number of insect pollinators are associated with this crop. Amongst them, honey bees are the most predominant. Pollination of crops by honey bees is one of the most practical and promising methods of increasing crop production[2]. Honey bees exhibit flower constancy and can forage for longer duration. Bees collect pollen and nectar to supply to the next generation, whereas other insects collect them for their individual needs only. Hence, bees make more flower visits. Their long tongues, coats of long collecting hair, and the ability to warm themselves and to work in cool weather make them generally more efficient pollinators than most other insects. Amongst honey bees, *Apis mellifera* L. is a commercial bee species which can be managed in sufficient numbers where and when required.

Orientation is a key behaviour shown by the bees. Whenever bees are kept in a new place (beyond flight range) or in confinement say in a net house, bees first orient and then start foraging. Past observations have shown that honey bees do not forage and work in net houses of smaller size. Hence, the present study was undertaken with the objective to primarily standardize the minimum dimensions (length × breadth × height) of the net house where bees can forage. Since information on the utilization of honey bees in cabbage seed production in net house conditions is not available, a preliminary attempt has been made to study the performance of *Apis mellifera* L. as pollinator in seed production of this crop.

Seed production of cabbage hybrid H-702 under net house conditions was studied during 2011–12 and 2012–13 at the Research farm of Department of Vegetable Crops and Floriculture, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur (altitude 1290 m amsl, between 32.11°N and 76.23°E). During 2011–12, cabbage was raised in a small ($l \times b \times h = 3 \times 2 \times 2.25$ m) and a large ($l \times b \times h = 15 \times 7 \times 4.5$ m) net house. During 2012–13, dimensions of the net house were $15 \times 7 \times 4.5$ m. For raising the seed, self-incompatible (SI) lines, i.e. I-4-6 and pollen parent, i.e. Golden Acre (Palampur strain) were used for producing seed. Table 1 provides details of materials used and methods employed.

During both the years of study, at the initiation of flowering in the crop, a queen right colony of *A. mellifera* of 1.5 bee frame strength was placed in one corner of each net house. The net house was maintained insect-proof from the very beginning of planting of the crop so that insects from the outside could not enter inside and bees were forced to remain confined in the net house. Data on foraging of bees were recorded in both the net houses (small and large) during 2011–12 and in a large net house during 2012–13. Besides, bee activity, including number of pollen and nectar gatherers, outgoing bees at the hive entrance (per 5 min) and the density of bees in 1 m^2 of the cropped area at the peak of flowering were also recorded at different time intervals (between 10.00 and 10.30 am (T_1), 12.00 and 12.30 pm (T_2), 2.00 and 2.30 pm (T_3) and 4.00 and 4.30 pm (T_4)) for six days. After the crop was ready, yield data were recorded during both the years of study.

During 2011–12, bees did not forage in the small net house and remained resting on the net house walls. No bee was observed to forage in this net house even up to five days, and some of the bees were observed to die in the net house. However, in the large net house bees were observed to rest on the net house walls for one day, but on the second day of placing of the bee colony, bees started their foraging activity normally. Foraging behaviour of bees was similar in both the years of study.

During 2011–12, the number of pollen-gathering bees was counted to be 34.67 ± 8.62 at T_1, which increased to 37.67 ±