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IDENTIFICATION OF BIRD REMAINS AFTER BIRD-AIRPLANE COLLISIONS, BASED ON DNA SEQUENCE ANALYSIS.

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Summary

In order to establish a method for the identification of bird remains after bird-airplane collisions, a technique was developed to isolate DNA from minute tissue samples, blood smears or feather fragments. PCR amplification of part of the mitochondrial cytochrome-b gene and sequence determination of the product allows identification of the origin of the sample to the species-level.

Since no data of cytochrome-b sequences of the bird species most frequently encountered in bird-airplane collisions were available from the GenBank database, the cytochrome-b gene of twenty species was partially sequenced and deposited into the database. For this purpose DNA was isolated from tissue of frozen specimens, feathers and blood samples, as well as from bird skins in the collection of the Zoological Museum Amsterdam.

Key Words. Identification, DNA Sequencing

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Introduction.

In bird-airplane collisions birds are either completely shattered against the outside of the plane or sucked into the engine, where turbine blades and high temperatures subsequently cause complete destruction. In many cases hardly any morphologically identifiable remains are left. In order for bird-strike statistics to reflect the actual situation, the identification of remains from bird-airplane collisions is crucial. In more than ninety per cent of cases identification based on microscopic feather analysis is possible to the family-level (Brom, 1986, 1991). However, for recognition of species this method depends on the presence of macroscopically identifiable feather-material. Identification of bird remains based on DNA-sequence analysis is expected to offer a valuable addition to the other identification techniques. The sequence of nucleotides in a stretch of DNA holds enough information for the identification of its origin down to the species level.

Since the quantity of DNA isolated from minute tissue samples, blood smears or feather fragments is insufficient for sequence analysis, the target sequence must be amplified first. The Polymerase Chain Reaction (PCR) offers the possibility to selectively amplify part of a DNA sequence bordered by two synthetic oligonucleotides termed 'primers' (Saiki *et al.*, 1988). The primers are derived from conserved parts of known DNA sequences, making it possible to amplify DNA from species for which there is no sequence information. Ideally, the same pair of primers should be able to amplify DNA originating from a great variety of bird species. Universal primers that amplify both bird and insect DNA are of importance too, as insects may be another major cause of pollution in airplane motors.

Owing to its circular nature and presence in a separate cellular compartment, mitochondrial DNA is thought to be more resistant to degradation than nuclear DNA, and was therefore chosen as the target molecule for the PCR amplification.

In this paper we describe briefly the method for isolation of DNA from minute samples of tissue, blood or feathers and amplification and sequence determination of a part of the mitochondrial cytochrome-b gene. For these purposes primers targeted at the mitochondrial cytochrome-b gene (only birds) and at the 16s ribosomal RNA gene (universal) were selected. Partial sequences of the cytochrome-b gene of the twenty bird species most frequently encountered in bird-airplane strikes were determined and deposited in the GenBank database.

Material and methods.

DNA-ISOLATION.

Samples of muscle tissue (20 mg), skin tissue (5 by 5 mm), or blood (25 µl) from frozen specimens, pieces of skin from museum specimens (5 by 5 mm), or samples of debris from airplane motors (varying quantity) were all treated in the same way: In a small reaction vial 100 µl of extraction buffer (100 mM Tris pH8.0; 10 mM EDTA; 100 mM NaCl; 0.1% (w/v) SDS; 1% (v/v) diethyl pyrocarbonate; and 0.5 µg/ml proteinase K) was added to the sample. After homogenisation of solid samples with a glass rod, the vials were incubated at 37°C for 3 hours. The lysed samples were then purified by extraction with an equal volume of Tris-saturated phenol (pH 8.0). After careful but thorough mixing and separation of the phases by spinning for one minute at full speed in a tabletop centrifuge (13,000 rpm), the water phase was removed to a new vial. The phenol phase was re-extracted with 100 µl water and both water phases were pooled and extracted with equal volumes of phenol/chloroform/iso-amyl alcohol (25/24/1) and chloroform/iso-amyl alcohol (24/1). Nucleic acids were pelleted from the final water phase by adding a half volume of 7.5 M ammonium acetate and two volumes of ice-cold absolute ethanol. Samples were immediately centrifuged at 13,000 rpm for 30 minutes at 4°C. After careful removal of the liquid by pipetting, the pellets were air-dried for 30 minutes and dissolved in 50 µl of distilled water. Yield and purity were estimated after agarose gel electrophoresis of 5 µl of the preparations.

PCR AND PURIFICATION OF PCR PRODUCT.

PCR amplification of 1 µl of the DNA preparations was done in 25 µl volumes in an Omnigene Temperature Cycler (Hybaid), using 0.1 µM of each primer, 0.2 mM of each dNTP and 0.5 units of *Taq* polymerase (SphaeroQ) per reaction, in the buffer prescribed by the manufacturer. Primers used in this study are listed in table 1. Cycling conditions were as follows: after an initial denaturation period of 2 minutes at 95°C, 5 cycles of 30 seconds at 95°C, 1 minute at 55°C, and 1 minute at 72°C were done, followed by 30 cycles of 30 seconds at 95°C, 1 minute at 60°C, and

conditions. These primer pairs were therefore omitted from further studies. The PCR products from primer pairs cytB-L / cytB-H and 16sX1 / 16sX2 were purified and their base sequences determined as described. Sequences from both primer pairs of all four species aligned well with the corresponding parts from the *Gallus gallus* total mitochondrial DNA sequence that was extracted from the GenBank database. This was taken as evidence that the PCR products were indeed cytochrome-b and 16s rDNA sequences. Furthermore, although phylogenetically widely separated, all four species were equally well amplifiable with both primer pairs, indicating the universal applicability of the primer pairs within the taxon birds.

To test the procedure for its applicability, four small samples of debris of unknown origin were taken from the motor of an F16 airplane after several hours of flight. Although no bird collision had been reported by the pilot, parts of the engine showed traces of debris. These samples were treated as described, and the isolates were used directly for PCR amplification, without prior yield-estimation. Only with primers 16sX1 and 16sX2 a product band of the expected size was found. Primers cytB-L and cytB-H did not form any product. Primers 16sX1 and 16sX2 were designed for black flies (Xiong and Kocher, 1991), but can be used for amplification of bird DNA too, as was determined with the program Amplify (Bill Engels, University of Wisconsin). As the cytB-L and cytB-H primers were adapted specifically for birds, the samples were not expected to be of bird origin. After sequence determination of the four samples and a GenBank database search for matching sequences, all 'hits' were 16s rDNA sequences from insects. This clearly showed that insects are a source of engine pollution in airplanes, causing slow accumulation of debris on motor parts.

Since our main interest was in identification of bird remains, cytB-L and cytB-H primers were chosen for further use in these studies. Furthermore, most bird sequences in the GenBank database are cytochrome-b sequences. Trying to identify samples of bird origin, we encountered the problem that for the birds most common in Western Europe no sequence data were available in the database. In order to be able to identify bird remains, we therefore set out to partially sequence the cytochrome-b gene of the 20 bird species most frequently found in bird-airplane collisions, and deposit these into the database (Table 2).

Nr.	Species	Source*
1	*Apus apus* - Swift	M
2	*Vanellus vanellus* - Lapwing	M
3	*Larus ridibundus* -Black headed gull	M
4	*Alauda arvensis* - Skylark	B
5	*Buteo buteo* - Buzzard	B/F
6	*Hirundo rustica* - Swallow	M
7	*Fringilla coelebs* - Chaffinch	M
8	*Columba livia* - Rock Dove	M
9	*Sturnus vulgaris* - Starling	M
10	*Falco tinnunculus* - Kestrel	M
11	*Larus argentatus* - Herring Gull	M
12	*Columba palumbus* -Wood Pigeon	M/S
13	*Turdus pilaris* - Fieldfare	B/S
14	*Anas platyrhynchos* - Mallard	M
15	*Larus canus* - Common Gull	M
16	*Delichon urbica* - House Martin	B
17	*Turdus iliacus* - Redwing	M
18	*Haematopus ostralegus* - Oystercatcher	M
19	*Turdus philomelos* - Song Thrush	M
20	*Ardea cinerea* - Grey Heron	M

Table 2. The twenty bird species most frequently engaged in bird-airplane collisions. Arrangement descendently in order of identified collisions.

* Origin of tissue used for DNA isolation: B. Blood; F. Feathers; M. Thoracic muscle; S. Dried skin. All samples except the blood samples were obtained from collections at the Zoological Museum of Amsterdam. Blood samples were obtained from the Avian Tissue Collection at the Zoological Institute of the University of Copenhagen, Denmark (Arctander & Fjeldså, 1991).

The reported study shows that possibilities exist to identify birds by means of DNA amplification from small fragments. The method has tentatively been applied to four samples of debris collected from airplanes and previously identified by microscopical study of feather fragments. In order not to let the senior researcher be influenced by any previous knowledge, these samples had been taken blindly from the collection of bird remains received during the last ten years. Unfortunately, it appeared that they all belonged to the passerines, order Passeriformes, of which the recorded database is still very fragmentary. In consequence, the sequenced samples could not be matched exactly with any species present in the database.

This state of affairs illustrates both the strength and the weakness of the method. A DNA-sequence is highly specific, but can only lead to identification when material of the species has been sequenced before. This means that the value of the technique will increase with time, but that a complete search leading to a specifically identified bird may for the moment be justified only when such an identification is vitally important, e.g. in cases where a plane has been lost in the accident under consideration.

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6 DISCUSSION ON STATISTICS AND IDENTIFICATION PAPERS

Mr Arie Dekker, The Netherlands, asked why swifts, being such small birds weighing only 45g caused so much damage. Is it the density of the birds?

Dr John Allen, UK, replied that it is not known but during the next summer their relative densities will be measured in co-operation with the US Department of Agriculture. Results should be known in the Autumn.

Dr Jan Wattel, The Netherlands, Identification WG Chairman, replied that perhaps the problem was related to the altitude as swifts fly high where the aircraft speed would be greater and therefore the impact more severe.

Mr Dekker replied that he didn't think it was the altitude as military aircraft fly very fast at low altitude, it must be something in the bird.

Mr John Thorpe, UK, commented that swifts had penetrated the windscreens of light aircraft and helicopters and had ended up, in one case, flying around inside the helicopter. And we must never overlook the problem of swifts and that they do fly fast and comparatively high and on a summer's evening will quite happily fly round, over and under a light aircraft. They avoid a light aircraft very nicely, because to see them is nearly impossible. By comparison in the motor glider in Israel where the birds had a 2 metre wingspan and were very hard to see even though they were large birds.

As IBSC Chairman he then went on to congratulate all the presenters on the quality and content of their presentations and that he and the Vice Chairman had commented at coffee break on the high standard and the way in which things had been put over in a very clear fashion with excellent illustrations.

Mr Jerry Yashon, Israel, commented that Ben Gurion Airport had been having some problems with bird strike reports, that aircrew seem to take it for granted that bird strikes with non-serious consequences don't need to be reported. They have been getting some very late reports through stewardesses who were not necessarily from the aircrew about incidents. He commented that keeping statistics is a very important and helpful manner in determining where the threat is coming from and how properly to allocate the resources available. So any message that can be sent out from IBSC to aircrew letting them know the importance of reporting bird strikes, when they happen, to the tower so that the bird strike team at the site can be on hand to take any sort of remains that are on the runway or at the site and make an immediate response because there may be ongoing bird activity there. Could IBSC put out some sort of notice to all the air operators the importance of reporting bird strikes when it happens at the site that it happens. The WG Chairman agreed.

Mr Olavi Stenman, Finland, asked Mr Christian Aas on his paper about military flying. What is the situation concerning fighter flying in north of Norway, very near the great bird colonies, when Mr Stenman had been there he had observed fighters flying very near the bird colonies causing trouble to the birds and a danger to themselves.

Mr Christian Aas, Norway, replied that the fighter pilots and other military pilots are warned about bird concentration areas and that they should avoid such areas if possible.

The WG Chairman commented that the result of many years of propaganda from IBSC and its local representatives to make all flight people more sensitive to the bird problem.

Dr Yossi Leshem, Israel, Statistics WG Chairman, commented that in Israel there were problems with helicopters training near colonies of Griffon vultures whenever the helicopter went close to the birds they chased them out of their nests and reproduction was down 50%. The chief of staff of the Israeli Air Force decided about 5 years ago to stop flying near the 3 most important colonies, although the airspace in Israel is very limited, due to pressure from conservationists.

Mr Dekker asked Dr Allan how he compares the bird strike prevention actions on different airfields, is the amount of effort for prevention put into an airfield measurable? How would it be quantified?

Dr Allan answered that an objective score based on the percentage of the staff involved in bird control that have been properly trained, level of equipment provision in terms of shell crackers, shot guns etc, provision of distress call equipment and also very importantly on the quality of the habitat management in terms of the proportion of the airfield that has bird repellent long grass, whether there are problems with trees growing on the airfield that may carry berries in the winter etc. Thus there is a series of objective scores and it is not based on personal opinion. Each factor is scored on a defined scale and a total percentage scored. If it clears the 70 level it is regarded as an acceptable investment of effort. This is looked at briefly in the paper. It is felt that each country would have to develop the idea in their own ways as different systems are used more heavily in different countries. Therefore CSL are not suggesting that theirs is the only system to be used but that there should be an objective way of assessing what is being done at aerodromes. Because of this, there is now a change in policy on behalf of the UK Civil Aviation Authority where each aerodrome is now required to produce a management statement saying exactly how it will achieve it's bird control objectives, how many of its staff will be trained etc and that statement is audited by the CAA and approved. It is then up to the aerodrome management to carry that out and they can be inspected on the standards they have set themselves. The statement is part of the certification of the aerodrome.

Mr Thorpe commented that the aerodrome is 'licensed' rather than certified. The CAA aerodrome inspector who deals with bird hazards and aerodrome audit etc was not attending the meeting because he was at a public enquiry at Edinburgh Airport in Scotland because someone had the intention of locating a garbage dump near the airport and it was felt that the public enquiry was more important for his evidence than him attending the meeting.

Sqn Ldr Roger Teegood, UK RAF, commented that his prime interest is on and around airfields and there is a separate organisation, the Directorate of Flight Safety who is dealing mainly with the low flying problems that have been encountered. The RAF will employ the system covered by Dr Allan as a means of quantifying and gauging the efficiency of those bird control units employed.

Dr Wattel then asked the audience for any comments on the system developed in the UK for assessing the level of bird strike prevention on airfields, have there been any changes to it for other countries or are there any plans to make changes for their country. Is it possible that it has been overdone and there is too much for people to take proper notice of it.

Dr Allan stated that people may think it is too complex or not complex enough, however, the system was designed and developed specifically to allow it to be applied in other countries. There are areas, such as the way aerodromes are scored for efficiency, definition of priority group bird species that will be different for other countries but it can be adapted and the simple statistics are easy to calculate and do not require any great statistical expertise, they are simple measures and they can be used to evaluate bird control efficiency. At what level you set your cut off points in terms of what is and is not acceptable is up to the country concerned. The view is that simple totals of bird strikes are not meaningful and can be harmful in terms of discouraging people from reporting.. It is known from Israel the need for all bird strikes to be reported. This system has been designed specifically to encourage reporting and to penalise poor reporting because poor reporting reflects on the airfield as a bad bird strike statistic as it brings down the proportion of small birds and that is why it has been done. It applies equally well in any country in Europe as well as it does in the UK. The crucial questions that you have to ask yourself before trying to apply these statistics are - do you have enough data and do you have a good enough reporting system in your country to make any of these statistics actually reliable - if you do then you can use them, if you don't you should be looking, as a national organisation, to improving your level of reporting, firstly so you can gather enough data to enable you to make a sensible evaluation.

Dr Wattel thought that reporting is vital to the whole bird strike concept and quite a bit can be improved on that. The working group would like to make a recommendation that most countries try to double their effort in getting the reporting rate at an acceptable level. A lot of countries have a very poor reporting rate and there is a lot of improvement possible. It has now been seen what good reporting standards can achieve.

Dr Leshem commented that it took 5 years to convince the Israeli Air Force to invest in the identification of feather remains that Judy Shamoun was working on. The thing that convinced the Air Force was the Royal

Netherlands Air Force data, showing how they got 88% of bird remains identified and the Israeli Air Force were getting 17% and the Middle East wanted to be like the Western Europeans. Therefore if you have data from other countries for a global picture this helps in your own small corner because the decision makers like to see what is going on. It takes some time but finally they will be convinced.

Mr Baron Rochard, UK, added his comments on the system which Dr Allan described. It is basically the logical end point of a paper that was produced by Tim Milson several years ago. The idea of being able to analyse bird strike statistics at greater depth and look at what sort of birds are being reported. He said that he uses this system within his company as a management tool. They run bird control units on RAF stations and monitor the performance of those units both on how well they are controlling birds, again by the proportion of preventable bird strike species and also monitor how well they are reporting bird strikes by the proportion of small birds like skylarks, swifts and swallows. They have been doing this for several years now and find it a perfectly practical management tool and a practical system to use.

Dr Wettel then invited others to comment on their systems, eg the ICAO system, also the EUROBASE for military aircraft, was there any link between these 2 or could they be mutually supportive, is there any contact, how is it maintained, do they work together, is it possible to analyse in similar ways?

Mr Alistair Pinos, ICAO, said that ICAO collect bird strike data on civilian aircraft, they do accept military strikes provided they occur on a civilian installation. They have resisted including military bird strikes in the data base because they are charged with collecting data on civilian aircraft, they are also charged with regularisation of civilian aviation and not military. They understand that there is always a need for co-ordination.

Dr Wettel asked if the ICAO and EUROBASE data bases were compatible in any way.

Mr Pinos replied that he did not have any information on the layout of the data. He would be able to supply a copy of the layout of the ICAO data base and bird strike information to the military authorities if they requested it. The ICAO data base is laid out to coincide with the ICAO reporting form.

Mr Thorpe commented that in 1972, when the Statistics WG was formed and he was put in charge as the chairman, he made it very clear that the military and civil data were separated out because the military aircraft are built to entirely different standards, their method of operation and mode of operation is quite different and therefore the simplest thing was to have 2 separate data bases and the only thing that you looked at was the birds because they were the common element. Also in the military world you often don't get the information about flying hours, flying movements etc because there has to be a degree of military secrecy. Therefore he felt it was better keeping the data bases separate and just looking at the similarity which is the birds, who can't tell whether they are being hit by a military aircraft or a civil one.

Dr Leshem suggested 2 recommendations for the WG. One is, as EUROBASE with the initiation of the RNLAF already have 13 different air forces using it by the next meeting in 1998 they should reach maybe 20 or 25 air forces using their data base. Arie Dekker has an ambition to come to 25 air forces, it will be then much easier for us to get access to the data.

The other point is for civilian statistics, if there is a simple formula that can be changed within the computers between the different countries so that the formula will be the same for all countries and they will be able to manipulate the data with the same data base it would be much easier then to move it from place to place. With communication through the internet and e-mail, this could be the next step for 1998.

The evaluating of the course is another most important tool to have also on the data that is being collected some of the air forces or the civilian airlines are not too keen to give the data but it is the ones who are open to give it who can help.

Ms Judy Shamoun, Israel, who deals with feather identification and statistics suggested that for the next meeting, the statistics papers should mention the feather identification methods used. There will be a difference in statistics with different techniques. If feather remains are only identified macroscopically rather than microscopically most small birds will fall out of the statistics, so it would be more interesting to know what type of techniques are being used.

Dr Wattel commented that it was very important that attention is paid to the identification even if you think it too expensive to send it out somewhere, try and do it yourself and record how you did it. He feels that identification is still a weak point in some places of the world. It would help in raising the standard of reporting.

Mr Thorpe would like to support the need for better identification, it has been said for years but now we have the method for doing it. One of his papers for Thursday will be on UK Transport Aircraft Turbine Engines. In the 94 cases of damage to an engine, we only have identification of the bird species in about 60% of cases. The work in the United States on large fan engine ingestion problems has again shown up exactly the same thing. This is something that everybody must address very strongly, this should even be aimed at the maintenance staff who pull the engines apart.

Dr Wattel commented that it is now possible for a 2 step identification process. You can try at the airport or at the nearby laboratory where microscopes are available to identify the microscopic remains using the expert system that will be for sale later this year. There will always be some cases where you hesitate, the centres at Tel Aviv and Amsterdam will be able to help out at reasonable costs, no more than they pay for the scientists doing the work also there is the expertise that would be difficult to build overnight in any other place.

Mr Tom Alge, USA, who is an accident investigator and investigates bird strikes said that sometimes when doing an investigation of a damaged engine there are no feathers available, sometimes they've gone on air turn-back and the feathers have blown away. You are lucky if you get 60% from the inspectors in the field. On investigations he thought that out of 20 investigations he had feathers for 10, the other 2 examined with black light, looking for debris and no feathers were found, therefore 60% sounds like a good statistic for finding feathers, especially after damage. The ones he investigates are the ones with major damage and probably a pretty big bird involved, but 10% of the time feathers will not be found.

Dr Allan recommended to the meeting the system used in the UK on the cost of bird remains identification. The services as remains identification experts are provided free to UK aerodromes in so far as the UK Civil Aviation Authority pays the bill for all the time involved in identifying bird remains. The same applies on the military side where the RAF picks up the bill centrally, there is no cost to the particular air station of submitting remains. He thinks that it is important that there are no barriers in the way of encouraging people to submit remains and in the UK at least there is no financial penalty involved to the aerodrome in actually sending this material in for expert identification.

Dr Wattel said that the same system applied in Holland but people who do most of the identification themselves can't expect the Dutch Civil Aviation Service to pay for their identification so they will have to pay for their identification so there will have to be a scheme developed of getting a bill sent to them but it will only be in a small percentage of cases where there is a great interest at stake or there is doubt whether the identification was the right one.

Ms Willemijn Prast, The Netherlands, asked about looking for feathers after a bird strike, you only need one tiny barbule which is almost invisible to the eye. This can identify the family or the order of the species of bird.

Mr Alge said that they do look for tiny pieces of feather, hopefully with some down on it. A strong white light is used, the engine is taken into the shop and a black light which is ultra-violet, is used, this shows the bird stains, enhances what is there and shows where the bird went in and how many birds. In the area where the bird(s) were ingested they look for any pieces of feather that they can find, no matter how small is bagged and tagged and identified where in the engine it was found.

Dr Wattel commented with the technique developed we can do it without feathers but that will be another 10 years before it is fully in operation.

Mr Rochard endorsed the above saying that downy fragments of feathers are almost always found after a bird strike, they almost always adhere even though they are not visible to the naked eye. Having looked some statistics of UK bird strike identifications and just for interest, UK civil airports have a rate of unidentified bird strike remains of 35% and for RAF stations - coastal 13.9% and inland 5%. Therefore it is possible to identify most the of the bird remains. It is easier to identify on-airfield strikes than military low-level en-route strikes because less will adhere to the aircraft en-route.

Mr Tim West, UK, said that on a US air base he had been working on they have had 100% identification for the last 3 years and that includes wiping some feathers off an aircraft that had been on there for over 1 month. Even if there is only blood visible it is soaked with water, wiped off and searched for feather remains, normally something is able to be found even if it is not visible to the naked eye.

Mr Donald Caccamise, USA, added information on the DNA methods. He realised that it would be more trouble than most people would be willing to go through but this material was very stable and can be collected, put in the freezer and saved, literally, for decades if it is handled properly and in a decade when DNA methods are available to us retrospective analyses of bird identifications can be done.

Mr Steinman, while editing the Green Booklet, had noticed that there are still countries who do not report bird strikes that do not cause damage. They are not included in the statistics at all. Please include in the recommendations that all bird strikes should be included in the statistics.

Dr Wettel fully supported this view.

The WG chairman closed the session by thanking everyone for their contributions.