J Ornithol (2013) 154:587–595
DOI 10.1007/s10336-013-0932-9

TECHNICAL NOTE

Factors affecting DNA quality in feathers used for non-invasive sampling

Nóra Vili · Edina Nemesházi · Szilvia Kovács · Márton Horváth · Lajos Kalmár · Krisztián Szabó

Received: 2 March 2012 / Revised: 18 December 2012 / Accepted: 11 January 2013 / Published online: 7 February 2013

Abstract The development of genetic methods broadens the scope of non-invasive sample types. Large shed feathers are good material for genetic analyses, since they are easy to collect and a single feather can provide a sufficient amount of DNA for PCR-based methods. Previous studies have demonstrated that feather quality and type affect the DNA quality extracted from the feather tips. Besides the tip, the superior umbilicus part of the shaft is also proposed as an appropriate source of DNA. In our study, we examined whether some feather parameters (physical condition, type and size) and storage time affect amplification success of DNA extracted from the superior umbilicus of shed Eastern Imperial Eagle (*Aquila heliaca*) feathers. We also tested the effects of sunlight, temperature and humidity on DNA extracted from Domestic Goose (*Anser anser domesticus*) feathers with amplification of fragments of various sizes, modelling the environmental conditions of the moulting season. While good quality feathers usually provided sufficient DNA, the usability of the DNA extracted from moderate quality feathers were affected by feather type. DNA quality was influenced in order of importance by humidity, direct sunlight and heat. Our findings support the usability of DNA samples derived from the superior umbilicus of shed feathers, and help to schedule field work by careful consideration of our results about feather quality and environmental factors.

Keywords *Aquila heliaca* · *Anser anser* · Shed feathers · Non-invasive sampling · DNA quality · DNA fragmentation

Communicated by M. Wink.

N. Vili (✉) · E. Nemesházi · K. Szabó
Department of Ecology, Institute for Biology, Faculty of Veterinary Science, Szent István University, Rottenbiller u. 50, Budapest 1077, Hungary
e-mail: Vili.Nora@aotk.szie.hu

S. Kovács
Department of Biomathematics and Informatics, Faculty of Veterinary Science, Szent István University, István u. 2, Budapest 1078, Hungary

M. Horváth
MME BirdLife Hungary, Species Conservation Group, Költő u. 21, Budapest 1121, Hungary

L. Kalmár
Institute of Enzymology, Hungarian Academy of Sciences Biological Research Center, Karolina út 29, Budapest 1113, Hungary

Zusammenfassung

Faktoren, die die Qualität der DNA aus Federn für nicht-invasive Probennahme beeinflussen

Die Entwicklung genetischer Methoden erweitert die Möglichkeiten zu nicht-invasiver Probennahme. Große Mausefedern eignen sich für genetische Analysen besonders, da sie leicht gesammelt werden können und auch eine einzelne Feder eine ausreichende DNA-Menge für die PCR-basierte Methoden liefern kann. Frühere Studien zeigten, dass die DNA-Qualität von der Qualität und dem Typ der Federn beeinflusst wird, wenn sie aus der Federspitze isoliert wird. Neben der Federspitze wird auch der obere Nabel der Feder für eine DNA-Isolierung verwendet. Wir untersuchten, ob die Amplifikation der aus dem oberen Nabel der Mauserfedern von Östlichen Kaiseradlern (*Aquila heliaca*) isolierten DNA durch Parameter wie Zustand, Typ, Größe und Lagerzeit der Feder beeinflusst ist, Auch prüften wir Mittels Modellierung der Umweltfaktoren während der Mauserzeit den Einfluss von Sonnenlicht, Temperatur und

Feuchtigkeit auf die aus Hausgansfedern (*Anser anser domesticus*) isolierte DNA durch Amplifikation von Fragmenten verschiedener Länge. Während Federn von guter Qualität im Allgemeinen genügend DNA lieferten, war die Benutzbarkeit der DNA aus Federn mäßiger Qualität von der Art der Feder beeinflusst. Die DNA-Qualität war in der Reihenfolge ihrer Wichtigkeit zudem von Feuchtigkeit, direkter Sonneneinstrahlung und Hitze beeinflusst. Unsere Ergebnisse unterstützen die Verwendbarkeit der aus dem oberen Nabel der Mauserfedern isolierten DNA-Proben und erlauben bei sorgfältiger Berücksichtigung unserer Ergebnisse zu Federqualität und Umweltfaktoren die Planung entsprechender Feldstudien.

Introduction

In recent years, the use of DNA-based methods has become more frequent in ecological studies (Haig et al. 2011), e.g. DNA can be used for genetic tagging of individuals for capture–recapture studies (e.g. Solberg et al. 2006; Lukacs and Burnham 2005; Mondol et al. 2009) or for the preparation and monitoring of species protection activities (e.g. Negro and Torres 1999; Crompton et al. 2008). As the application of non-invasive sampling techniques allows the studying of populations without the individuals needing to be captured, these methods have become more important in ecological genetics. It makes sampling easier and less stressful for the studied animals (Waits and Patkeau 2005; Beja-Pereira et al. 2009), and can be money- and timesaving. Furthermore, this method of sampling is the only possibility for genetic analyses in the case of several endangered and rare species, or when capturing is extremely difficult and could harm the individuals.

However, there are several serious limitations that coincide with the obvious advantages of non-invasive sampling. Most animal tissues sampled this way provide DNA of low copy number and quality (Taberlet et al. 1999). These are either dead tissues containing only a few damaged cells, or excrements that are often contaminated with foreign DNA, contain significant amounts of PCR inhibitors, and become digested quickly by endogenous nucleases (Monteiro et al. 1997; Murphy et al. 2003a, b; Nsubuga et al. 2004; Hájková et al. 2006; Beja-Pereira et al. 2009). Moreover, there are several uncertainties concerning the sampled specimens' species, sex or identity. In addition, the amount of the sample derived from a single individual can be a limiting factor when assigning individual DNA profiles (Gagneux et al. 1997; Reiners et al. 2011). However, with adequate laboratory practice and caution, DNA isolated from such samples can be sufficient for individual identification (Stoneking 1995; Taberlet and Luikart 1999; Waits and Patkeau 2005; Rudnick et al. 2005).

In contrast to hair, faeces and urine, shed feathers are usually easier to collect from individuals and yield better DNA (Harvey et al. 2006; Rudnick et al. 2005, 2007). Collection of moulted feathers of larger-sized birds such as pigeons (Seki 2006) or bigger is relatively easy compared to other non-invasive sampling methods, and in most cases the target species can be identified based on the feather (Rudnick et al. 2007). There are several methods published for DNA extraction from feathers (Taberlet and Bouvet 1991; Horváth et al. 2005; Bayard De Volo et al. 2008), and most commonly the feather tip is used (e.g. Hogan et al. 2008; Gebhardt et al. 2009). Another possible DNA source is the superior umbilicus part of the feather shaft (Horváth et al. 2005). This suggests higher yield and better DNA quality. This method is well cited; however, it has been applied in only a few studies, mostly involving large raptors since relatively large feathers with visible and isolatable superior umbilicus are needed (e.g. Seki 2006; Hailer et al. 2007; Martínez-Cruz et al. 2007; Banhos et al. 2008; Alcaide et al. 2010; Väli et al. 2010; Miller et al. 2011).

Hogan et al. (2008) studied feather tip samples of the Powerful Owl (*Ninox strenua*) in order to determine whether the type and condition of the feather affects the DNA yield. According to their results, the initial condition of the feathers is vital regarding the outcome of the studies while feather type has no influence on it.

The quality of non-invasively collected shed feathers depends greatly on field conditions (Johansson et al. 2012). High relative humidity, high temperature and sunlight can cause visible changes in feathers, but they are also known to damage DNA. Humidity causes the most noticeable physical damage, as the calamus becomes sponge-like, creating openings for various microorganisms. Murphy et al. (2003a) demonstrated that, after 60 days in a humid area, DNA quality extracted from bear faeces dropped considerably. The effects of heat and sunlight are more subtle. At higher temperatures, DNA was found to be more uncoiled and therefore more exposed to the effects of UV-B radiation (Li and Paulsson 2002), which is the most effective component of sunlight reaching the ground, although UV-A radiation may also be of importance (Ravanat et al. 2001).

The quality of the extracted DNA also depends on the storage conditions of the samples, because of the risk of high endogenous nuclease activity. Storage of faeces samples has been thoroughly studied and faeces are recommended to be stored at −20 °C in order to inhibit enzymatic activity (Wasser et al. 1997; Murphy et al. 2003a; Roeder et al. 2004). However, the effect of storage time and conditions has to be considered when also using samples like hair or feathers. Despite these sample types being less exposed to enzymatic processes, several external

factors (humidity, UV radiation and decomposers) can possibly influence DNA yield.

The most common method to determine the quality of DNA is to measure the concentration of the extracted DNA solution. This can be sometimes impractical, because the spectrophotometer also measures DNA, RNA, protein fragments and single nucleotides, and this can lead to overestimation of the DNA concentration (Teare et al. 1997). Furthermore, it does not provide information about the degree of fragmentation, which can be satifactorily predicted by the visualisation of the extracted DNA solution on agarose gels showing the extent of smearing. More accurate estimates can be made by the amplification success of different long fragments (Hogan et al. 2008; Zayats et al. 2009).

In our study, we tested the validity of the findings of Hogan et al. (2008) on large shed feathers, namely that good quality feathers yield better quality DNA and feather type is of less importance, provided the sample source is the superior umbilicus part of the feather shaft. We examined the effect of physical condition, feather type, size and storage time on shed feathers of Eastern Imperial Eagles (*Aquila heliaca*). We also investigated the effects of sunlight, temperature and humidity on fresh Domestic Goose (*Anser anser domesticus*) feathers and determined the most threatening environmental factors that have to be taken into consideration when collecting and storing shed feathers for genetic analyses.

Methods

Sample collecting and handling

Shed feathers of Eastern Imperial Eagles were collected between June and August in all years from 1997 to 2006 under active nests in East-Hungary (46°20′–48°35′N, 19°30′–21°35′E). Collected feathers were individually tagged, categorized (Table 1) and measured (calamus diameter, calamus and vane length); if calamus diameter was less than 1.5 mm, the sample was excluded from the study. Medium-sized miscellaneous feathers, like scapulars, primary coverts and alula feathers were grouped together since there was no difference in their average size (calamus diameter: $t = -0.5963$, $df = 93$, $p = 0.5524$ and calamus length: $t = 1.648$, $df = 93$, $p = 0.1024$, Welch tests).

Based on their physical appearance, feathers were ranked into three categories (Fig. 1): "poor", ($n = 6$) degraded, calamus in very poor condition, damaged superior umbilicus; "abraded", ($n = 81$) visible degradation of the calamus, slight degradation of the superior umbilicus; and "good", ($n = 494$) calamus in good condition, no

Table 1 Type, quality and calamus diameter of Eastern Imperial Eagle (*Aquila heliaca*) feathers examined in the study

Feather type	Quality (*n*)				Calamus diameter (mm)
	Good	Abraded	Poor	Total	
Primaries	122	23	1	146	5.5–8.9
Secondaries and tertials	137	20	1	158	3.3–6.7
Tail feathers	66	13	1	80	5.5–7.2
Medium size feathers[a]	102	11	2	118	2.0–5.3
Greater coverts[b]	67	11	1	79	1.9–5.2
Total	496	82	6	581	

[a] Scapular, primary covert, alula, >3 mm calamus diameter

[b] 1.5–3 mm calamus diameter

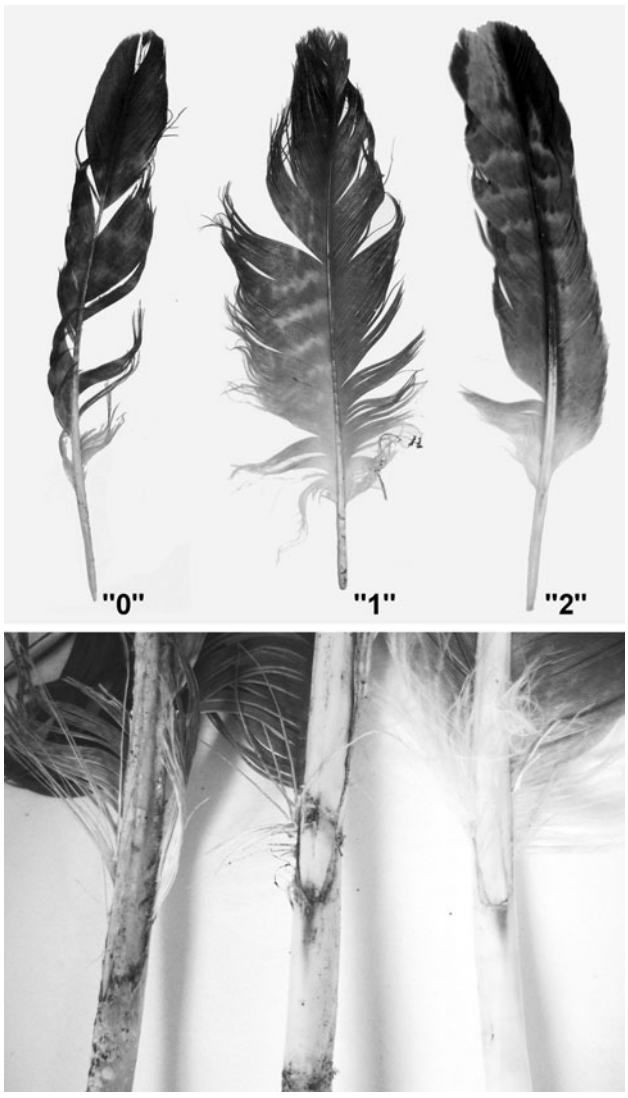

Fig. 1 Typical physical appearance of feathers belonging into three quality categories: poor (*"0"*), abraded (*"1"*) and good (*"2"*), with enlarged view of the superior umbilicus below

damage at the superior umbilicus. Feathers were stored in plastic bags under dry, dark and cool (room temperature or below) conditions until sample preparation.

Goose primaries were gathered at a goose farm that breeds parent stock for "Babat" Grey Landes geese in Babatpuszta, Hungary. To ensure that all feathers were in the same condition at the start of the experiments, feathers were plucked at the early phase of moulting, when their bases were already loose, so that feathers could be easily removed and geese were not exposed to significantly more stress than at a usual veterinary examination. The main difference between the usability of feather tips as a DNA source of plucked and moulted feathers is the condition of living tissue which sticks on the outer basal surface of the feather shaft. As the sampled superior umbilicus is not inside the skin, it was assumed that its quality is not different from freshly moulted feathers. Therefore, it can model the initial condition of large shed feathers in order to examine the effects of different environmental factors. Plucked feathers were stored in a freezer until the start of the treatments.

Treatments on goose feathers

Natural sunlight Feathers were attached to the bottom of a plastic box, covered with UV transparent foil (JK International GmbH), to exclude external humidity. To keep the feather completely dry previously desiccated silica gel granulates were placed in the box. After the experiment the silica gel was not saturated, so the interior of the box was assumed to be absolutely dry. The box was placed in September for 20 days on a southern facing window sill approximately 4 m high. During the time of the experiment the temperature varied between 12 and 23.6 °C.

Artificial sunlight In order to create monitored circumstances we repeated the former experiment on the effects of sunlight. We illuminated the feathers with a Repti Glo 5.0 (ExoTerra, 60 cm, 20 W) linear bulb which produces a spectrum including UV-B, UV-A, visible and infrared light. In order to keep the treatment comparable with the previous one, the length of the treatment was set to 100 h, which is approximately equal with the length of the average number of sunshine hours with the most intense solar radiation in September in Hungary (5 h/day for 20 days). In order to keep the feathers dry we used the same method as described above. To ensure that the light bulb is the only light source and to exclude exterior humidity the tray and the light source was covered with black nylon film. The temperature was constantly below 30 °C in the range 22–29 °C.

Dry heat Dry heat was produced in a laboratory oven (Memmert, ULE 500) set to a constant 30 °C, this is 2°–3° higher than the average summer temperature in Hungary. The experiment lasted 100 h. To keep the feathers dry, desiccated silica gel was placed next to them into the plastic bags containing the feathers.

Heat and humidity Feathers were placed into a water bath on a polystyrene tray, to avoid direct contact with the water. The temperature was set to a constant 30 °C temperature. The treatment lasted 100 h.

Disinfection in treatments "dry heat" and "heat and humidity" In the pilot study, we found mould-like discolouration on the feathers at the end of the experiment. In order to check the effects of the presumed microorganisms the surface of the feathers in these groups was disinfected (Descosept, Dr. Schumacher GmbH). Additionally, in these treatments both the water bath and the water used in it was thoroughly cleaned and exposed to UV-C light for 8 h. Dry heat was produced in the same laboratory oven as in the non-disinfected *dry heat* treatment group (Memmert, ULE 500) but it was previously sterilized on 170 °C for 1 h.

As the feathers could be placed into the instruments only after sterilization, the circumstances cannot be considered completely sterile. All other experimental settings were identical to the non-disinfected groups.

Control group No special treatment was applied, feathers were stored at −20 °C until sample preparation.

The superior umbilicus part of the feather shaft was cut immediately after the treatments (goose feathers) or within 1 year after collection (eagle feathers) and stored at −20 °C in a microfuge tube until DNA extraction. Sample preparation of both eagle and goose feathers was carried out as described by Horváth et al. (2005). DNA extraction was carried out according to the standard salting out method (Gemmel and Akiyama 1996). In order to facilitate the digestion process 10 µl dithiothreitol (DTT; 1 M) was given to each sample (Weigmann 1968).

DNA quality control To estimate fragmentation of the extracted DNA from both eagle and goose feathers, two methods were used: (1) extracted DNA solution was visualized by agarose gel electrophoresis, and (2) genomic regions of various lengths were amplified by PCR (Hogan et al. 2008; Zayats et al. 2009).

In eagle feathers sex-chromosome-linked CHD gene introns were amplified with the primers (*2550F/2718R*) and procedures described by Fridolfsson and Ellegren (1999). To test the results of the treatments on goose feathers three nuclear loci of small, medium and large sizes were amplified. Two microsatellite loci *Bcaµ6* (approximately 150 bp, Buchholz et al. 1998) and *Ans24*, (approx. 300 bp, Weiß et al. 2008) were chosen and partial sequences of the α-actin gene with the primers *Act2* and *Act4*, amplifying a 985 bp sequence (Rodríguez et al. 2003). All PCRs were carried out according to the published procedures.

At the electrophoretic visualisation, we used a three-grade scale for ranking the quality of the extracted DNA and all PCR products except for the CHD gene introns: ''not visible (0)'' means no visible DNA or PCR product; ''detectable (1) '' indicates clearly visible, but smeared DNA or visible but very light PCR product; and ''clear (2)'' represents well defined and clearly visible DNA or PCR product. For the CHD products, the scale was dichotomous because initially this reaction was used for testing the usability of eagle samples, meaning if this amplification failed the sample was omitted from further analyses: "unsuccessful (X)" represents indefinite PCR product or no product at all, and "successful (S)" represents amplifications with definite PCR product. DNA solutions were visualized on 0.8 %, PCR products on 2 % agarose gels.

Statistical analyses

Conditional inference trees were built to analyze the effects of physical condition, feather type, calamus diameter and storage time on eagle feathers, and to reveal the effects of sunlight, artificial light, temperature and humidity on goose feathers. Conditional inference trees are unified framework of recursive binary partitioning with piecewise constant fits statistically confirmed by permutation tests developed by Strasser and Weber (1999). The covariates measured at different scales are selected unbiased, and multiple test procedures are applied to determine whether the recursion needs to stop. Splitting criterion of the tree was set to $p = 0.2$ (in eagles) and $p = 0.1$ (in geese), that is, only the variables with p values less than 0.2 and 0.1 are represented in the tree. The criterion was defined so that the next node after the last significant ($p = 0.05$) difference was also represented. The minimum number of observations in a terminal node was set to 5 (eagle feathers) and 16 (goose feathers) as this was the sample size in each group. Test type was set to "Bonferroni" in order to get multiplicity adjusted p values (Strasser and Weber 1999). Statistical analyses were carried out with the R 2.12.1 statistical program (R Development Core Team 2011), and the package "party" was used to estimate the conditional inference trees (Hothorn et al. 2011).

Results

Eagle feathers: effects of physical condition, feather type, calamus diameter and storage time

Amplified PCR product could be defined in 2 of 6 cases (33.3 %) in the poorest quality group, in 66 cases (81.4 %) in the abraded group and in 429 cases (86.8 %) in the good quality group. Regarding the feather type, success rate ranged between 82.1 % (greater covert feathers) and 92.5 % (tail feathers).

Good and abraded quality feathers yielded less fragmented DNA than poor quality feathers (Fig. 2, first and second node, $p = 0.002$, and 0.018, respectively). In addition, amplification success of abraded quality feathers differed (although not significantly) according to feather type, with less definable PCR product in greater coverts, secondaries and tertials (Fig. 2, fourth node, $p = 0.121$).

As storage time and calamus diameter had no significant effect on amplification success, these variables are not represented on the conditional inference tree.

Goose feathers: effects of light, heat and humidity

Altogether, 112 feathers were used in the experiments, with 16 feathers assigned to each group. To get preliminary estimates on the DNA fragmentation, extracted DNA solutions were visualised on agarose gels and graded according to their degree of smearing.

The amplification of both the 151-bp-long *Bcaμ6* and the 300-bp-long *Ans 24* fragments yielded at least moderate quality PCR products in all groups. The 985-bp-long part of the *α-actin* gene could only be amplified in the control group and in groups treated with *dry heat* (with or without disinfection) and artificial sunlight. These results correspond with the preliminary expectations made after the electrophoretic visualisation of extracted DNA solutions.

The size of the amplified fragment was the first splitting factor on the conditional inference tree (Fig. 3). It divided the amplification success rates into two groups: large versus medium and small (node 1, $p < 0.001$). On the left branch, the control group was separated from the other groups (node 2) as it yielded slightly better results, although this difference was only marginally significant ($p = 0.072$). On the right branch, the group treated with *heat and humidity* had the poorest amplification success in both small- and medium-sized fragments (node 5, $p < 0.001$). At the next node (node 7), samples treated with *natural sunlight* had worse DNA quality ($p = 0.003$) than the samples stored in dark (i.e. no light treatment was included) or treated with *artificial sunlight*. Similarly to the previous splitting node there was no difference according to the fragment size. At node 8, the results were divided according to the size of the amplified fragment ($p = 0.008$). In medium-sized fragments, amplification success did not differ significantly among the groups, but it did in small fragments, according to the treatments. At the amplification of small fragments, the groups *disinfected dry heat* and the *control* yielded better PCR success with a marginally significant value (node 9; $p = 0.069$) than the groups *disinfected heat and humidity* and *dry heat*.

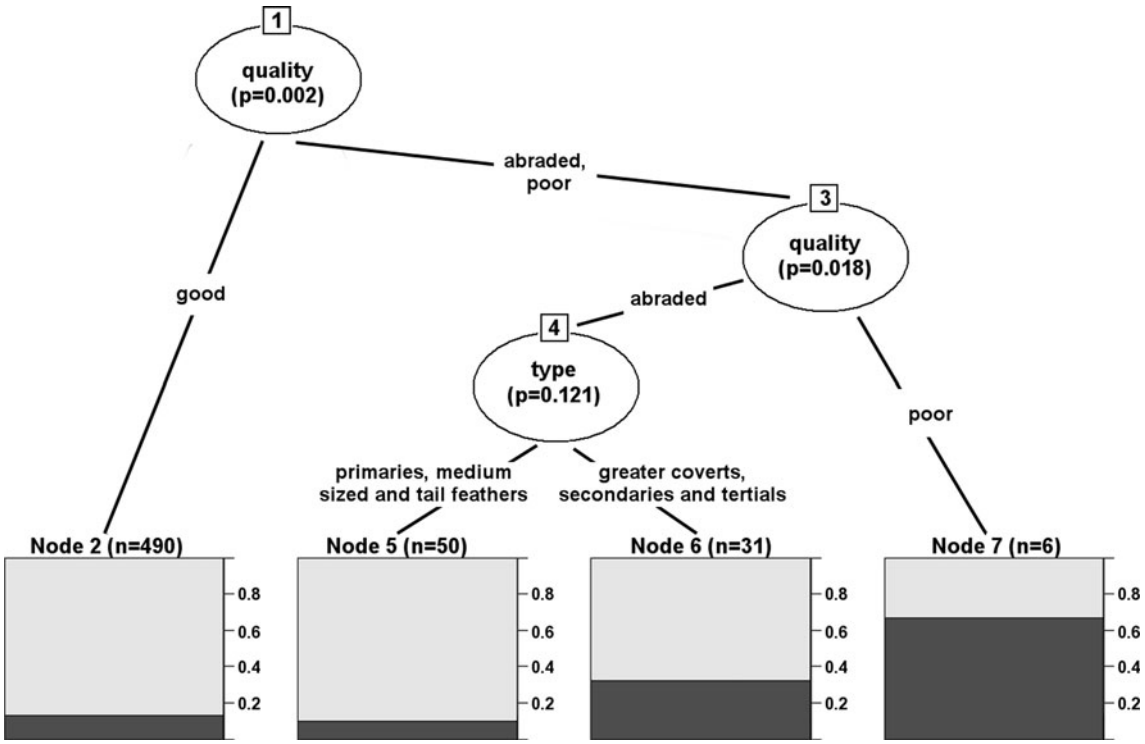

Fig. 2 Conditional inference tree for the effects of feather quality and feather type on the success of amplification of the CHD1 W/Z fragments from Eastern Imperial Eagle feathers. *p* values are shown at nodes *1*, *3*, *4*; *light grey* areas represent the rate of successful amplifications of the CHD1 fragment, *dark grey* areas represents the rate of failed amplifications; variable names are shown in the *circles*, sample sizes are represented on the *top* of the *boxes*

Discussion

Eagle feathers: effects of physical condition, feather type, calamus diameter and storage time

Our results confirm and broaden the findings of Hogan et al. (2008) about the importance of feather quality with the use of the superior umbilicus as DNA source. Even though feather type did not affect amplification success very much, the groups *primaries*, *medium sized feathers* and *tail feathers* yielded better results than *greater coverts*, *secondaries and tertials* as seen in Fig. 2. Although our results are not entirely clear on that aspect, we suggest using feathers from the first group when using superior umbilicus as DNA source. As feathers with calamus diameter below 1.5 mm were excluded from the analyses, it is likely that small feathers sampled at the superior umbilicus would yield less DNA and worse amplification results, as was found previously by Hogan et al. (2008). The fact that storage time had no significant effect on DNA quality can be explained by the storage conditions, as feathers were stored in a dry and dark room, and the removed blood clots were placed in a freezer (−20 °C) until DNA extraction.

Goose feathers: effects of fragment size, sunlight, heat and humidity

Estimating the level of DNA fragmentation via electrofo-retic visualisation of the extracted DNA solution was ambiguous. In the minor fragments (*Bcaμ6* and *Ans24*), amplification success was well predicted by the results of the DNA grading, but in the 985-bp *α-actin* fragment, amplification success was incidental. This result together with the basic stability data from full DNA electrophoresis implies that this evaluation method gives reliable estimates on the usability of samples when using short and medium length fragments.

Nevertheless, the amplification success was consider-ably affected by the size of the amplified fragment as the amplification success of the large fragments differed significantly from the medium and small ones (Fig. 3, node 1). In this large fragment length, any type of treatment had a negative effect on PCR success (although not significant), compared to the control group. Moreover, even in the control group, only a few samples gave an evaluable PCR product. This suggests that shed feathers are rarely suitable for the PCR-based analyses of such large fragments (985 bp), and even small impacts can largely reduce their

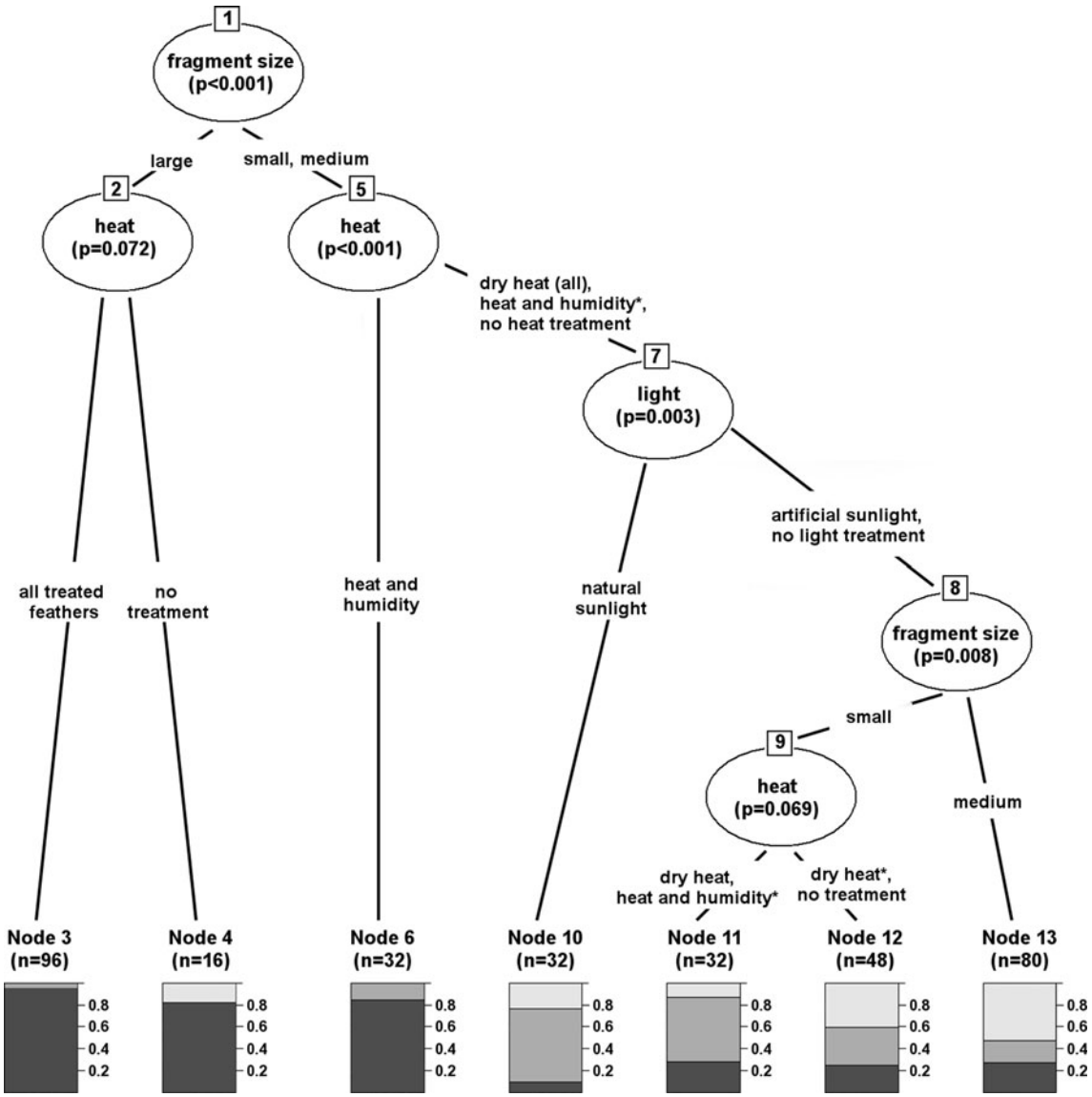

Fig. 3 Conditional inference tree for the effects of fragment size, heat, humidity and light on the amplification success of three nuclear loci (Act2/Act 4—partial sequence of the α-actin gene, 985 bp-, Ans 24–300 bp, Bcaμ6–151 bp) in goose feathers; *p* values are shown at nodes *1*, *2*, *5*, *7*, *8* and *9*; *light grey* area represents the rate of PCR product with good quality, medium *grey area* represents the rate of poor quality PCR products and *dark grey area* represent the rate of failed amplifications; variable names are shown in the *circles*, sample sizes are represented on the *top* of the *boxes*. *Asterisk* indicates disinfected feathers and experimental environment

usability. This agrees with other studies, which have also suggested a prefererence for small- or medium-sized fragments for non-invasive genetic tagging of animals (e.g. Taberlet et al. 1999; Broquet et al. 2006; Beja-Pereira et al. 2009) since genotyping error rates correlate with the fragment size (Hoffman and Amos 2005).

Among the treatment factors, humidity reduced PCR performance to the greatest extent. Amplification success of the smaller fragments (both medium and small) in the group treated with *heat and humidity* was significantly lower than in the groups with any other treatments (*dry heat with and without disinfection, heat and humidity with disinfection, artificial sunlight* and the *control*; Fig. 3, node 5). This poor performance can be assigned to the activity of decomposing microorganisms. Even in our experimental circumstances, mould spores and hyphae could be detected (stained with Cotton Blue dye) on humidity-treated feathers either in disinfected or non-disinfected cases, but not on *control* and *dry heat*-treated ones. This implies that surface disinfection is not sufficient to prevent DNA degradation. Since humidity can provide optimal circumstances for both fungi and other keratin-degrading microorganisms (Onifade et al. 1998), and change the texture of the rachis exposing the matrix (Lingham-Soliar and Bonser 2010), it

can be assumed that the blood clot was damaged by these microorganisms. Therefore, shed feathers exposed to wet (or warm and wet) environments for longer periods are expected to fail in DNA-based work.

Within the remaining groups, *natural sunlight* was the most important splitting factor (Fig. 3, node 7). Its negative effect on PCR success did not differ according to the fragment size, being present in both 150- and 300-bp range, suggesting that the sunlight can cause damage that also prevents the amplification of fragments below the 300-bp range. This means that feathers exposed to direct sunlight for a long time might be inefficient as a DNA source even if they are seemingly well preserved.

The fact that feathers treated with *artificial sunlight* had one of the best amplification results among all treatments was surprising, compared to the poor performance of the group treated with natural sunlight. It is possible that the proportion of components in the natural and artificial sunlight were different and that this resulted in less damage to the DNA in the latter case, since photosensitisation and the extent of DNA damage depends on the wavelength of light (Kielbassa et al. 1997; Ravanat et al. 2001).

Effect of fragment size was also present in the shorter PCR products. In the medium-sized 300-bp fragment PCR, success was independent of any treatment, but in the smallest PCR product, *dry heat* and *disinfected humid heat* resulted in slightly worse amplification success compared to the *control* and *disinfected dry heat* treatment, suggesting that heat and humidity deteriorates DNA even in the shortest fragments, and that disinfection can partly improve the results by hampering microorganisms.

Our study was carried out to discover the threatening environmental factors that influence the quality of DNA extracted from the superior umbilicus of large flight feathers. However, in the case of smaller feathers or sampling methods other than the superior umbilicus, we suggest following the screening protocol developed by Hogan et al. (2008) in order to achieve good quality DNA.

In conclusion, to get the best results when working with non-invasively sampled feathers, we suggest using good quality feathers that are collected as soon as possible after moult, especially in localities where they could be exposed to humidity and direct sunlight. In these samples, type and size of the feathers are of less importance, and when stored properly (in freezer or in a dark, dry and cool place), storage time can be prolonged. Using standard DNA extraction methods, the superior umbilicus proved to be a practical source of genetic material in the case of small- and medium-sized fragments. This is confirmed by our finding that moderate quality feathers provided nearly the same quality DNA as good quality feathers (81.4 and 86.8 %); although in the former case the feather type can affect the results. Nevertheless, even in ideal conditions, DNA extracted from these moulted feathers will be sub-optimal when amplifying large nuclear fragments.

Acknowledgments We would like to thank the Hungarian Imperial Eagle Working Group and the Anser-Branch Ltd. (Babatpuszta, Hungary) for providing the feathers and for their cooperation. We are grateful to Dr. Iren Siller, for her observations on the mould and to Dr. Andrea Harnos for her help in the statistical analyses. We also would like to thank Dr. János Kis and two anonymous reviewers for their valuable comments on the manuscript. All work described here complies with the current laws of Hungary.

References

Alcaide M, Cadahía L, Lambertucci SA, Negro JJ (2010) Noninvasive estimation of minimum population sizes and variability of the major histocompatibility complex in the Andean condor. Condor 112:470–478

Banhos A, Hrbek T, Gravena W, Sanaiotti T, Farias IP (2008) Genomic resources for the conservation and management of the Harpy Eagle (*Harpia harpyja*, Falconiformes, Accipitridae). Genet Mol Biol 31:146–154

Bayard De Volo S, Reynolds RT, Douglas MR, Antolin MF (2008) An improved extraction method to increase DNA yield from molted feathers. Condor 110:762–767

Beja-Pereira A, Oliveira R, Alves PC, Schwartz MK, Luikart G (2009) Advancing ecological understandings through technological transformations in noninvasive genetics. Mol Ecol Resour 9:1279–1301

Broquet T, Ménard N, Petit E (2006) Noninvasive population genetics: a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. Conserv Genet 8:249–260

Buchholz WG, Pearce JM, Pierson BJ, Scribner KT (1998) Dinucleotide repeat polymorphisms in waterfowl (family Anatidae): characterization of a sex-linked (Zspecific) and 14 autosomal loci. Anim Genet 29:323–325

Crompton AE, Obbard ME, Petersen SD, Wilson PJ (2008) Population genetic structure in polar bears (*Ursus maritimus*) from Hudson Bay, Canada: implications of future climate change. Biol Conserv 141:2528–2539

Fridolfsson A-K, Ellegren H (1999) A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol 30:116–121

Gagneux P, Boesch C, Woodruff DS (1997) Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair. Mol Ecol 6:861–868

Gebhardt KJ, Brightsmith D, Powell G, Waits LP (2009) Molted feathers from clay licks in Peru provide DNA for three large macaws (*Ara ararauna, A. chloropterus, and A. macao*). J Field Ornithol 80:183–192

Gemmel N, Akiyama S (1996) An efficient method for the extraction of DNA from vertebrate tissue. Trends Genet 12:338–339

Haig SM, Bronaugh WB, Crowhurst RS, D'Elia J, Eagles-Smith CA, Epps CW, Knaus B, Miller MP, Moses ML, Oyler-McCance S, Robinson WD, Sidlauskas B (2011) Genetic applications in avian conservation. Auk 128:205–229

Hailer F, Helander B, Folkestad AO, Ganusevich SA, Garstad S, Hauff P, Koren C, Masterov VB, Nygård T, Rudnick JA, Shiraki S, Skarphedinsson K, Volke V, Wille F, Vilà C (2007) Phylogeography of the white-tailed eagle, a generalist with large dispersal capacity. J Biogeogr 34:1193–1206

Hájková P, Zemanová B, Bryja J, Hájek B, Roche K, Tkadlec E, Zima J (2006) Factors affecting success of PCR amplification of microsatellite loci from otter faeces. Mol Ecol Notes 6:559–562

Harvey MG, Bonter DN, Stenzler LM, Lovette IJ (2006) A comparison of plucked feathers versus blood samples as DNA sources for molecular sexing. J Field Ornithol 77:135–140

Hoffman JI, Amos W (2005) Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. Mol Ecol 14:599–612

Hogan FE, Cooke R, Burridge CP, Norman JA (2008) Optimizing the use of shed feathers for genetic analysis. Mol Ecol Resour 8:561–567

Horváth MB, Martínez-Cruz B, Negro JJ, Kalmár L, Godoy JA (2005) An overlooked DNA source for non-invasive genetic analysis in birds. J Avian Biol 36:84–88

Hothorn T, Hornik K, Strobl C, Zeileis A (2011) Party: a laboratory for recursive partytioning, cran.r-project.org, http://cran.r-project.org/web/packages/party/index.html. Accessed 29 Aug 2011

Johansson MP, McMahon BJ, Höglund J, Segelbacher G (2012) Amplification success of multilocus genotypes from feathers found in the field compared with feathers obtained from shot birds. Ibis 154:15–20

Kielbassa C, Roza L, Ebe B (1997) Wavelength dependence of oxidative DNA damage induced by UV and visible light. Carcinogenesis 18:811–816

Li S, Paulsson M (2002) Temperature-dependent formation and photorepair of DNA damage induced by UV-B radiation in suspension-cultured tobacco cells. J Photochem Photobiol B 66:67–72

Lingham-Soliar T, Bonser RHC (2010) Selective biodegradation of keratin matrix in feather rachis reveals classic bioengineering. Proc R Soc Lond B 277:1161–1168

Lukacs PM, Burnham KP (2005) Review of capture–recapture methods applicable to noninvasive genetic sampling. Mol Ecol 14:3909–3919

Martínez-Cruz B, Godoy JA, Negro JJ (2007) Population fragmentation leads to spatial and temporal genetic structure in the endangered Spanish imperial eagle. Mol Ecol 16:477–486

Miller JM, Hallager S, Monfort SL, Newby J, Bishop K, Tidmus SA, Black P, Houston B, Matthee CA, Fleischer RC (2011) Phylogeographic analysis of nuclear and mtDNA supports subspecies designations in the ostrich (*Struthio camelus*). Conserv Genet 12:423–431

Mondol S, Karanth KU, Kumar NS, Gopalaswamy AM, Andheria A, Ramakrishnan U (2009) Evaluation of non-invasive genetic sampling methods for estimating tiger population size. Biol Conserv 142:2350–2360

Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R, Cabrita J, Mégraud F (1997) Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. J Clin Microbiol 35:995–998

Murphy MA, Kendall KC, Robinson A, Waits LP (2003a) The impact of time and field conditions on brown bear (*Ursus arctos*) faecal DNA amplification. Conserv Genet 8:1219–1224

Murphy MA, Waits LP, Kendall KC (2003b) The influence of diet on faecal DNA amplification and sex identification in brown bears (*Ursus arctos*). Mol Ecol 12:2261–2265

Negro JJ, Torres MJ (1999) Genetic variability and differentiation of two bearded vulture *Gypaetus barbatus* populations and implications for reintroduction projects. Biol Conserv 87:249–254

Nsubuga AM, Robbins MM, Roeder AD, Morin PA, Boesch C, Vigilant L (2004) Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method. Mol Ecol 13:2089–2094

Onifade AA, Al-Sane NA, Al-Musallam AA, Al-Zarban S (1998) Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresour Technol 66:1–11

Ravanat JL, Douki T, Cadet J (2001) Direct and indirect effects of UV radiation on DNA and its components. J Photochem Photobiol B 63:88–102

Reiners TE, Encarnação JA, Wolters V (2011) An optimized hair trap for non-invasive genetic studies of small cryptic mammals. Eur J Wildl Res 27:991–995

Rodríguez MA, García T, González I, Asensio L, Mayoral B, López-Calleja I, Hernández PE, Martín R (2003) Development of a polymerase chain reaction assay for species identification of goose and mule duck in foie gras products. Meat Sci 65:1257–2363

Roeder AD, Archer FI, Poinar HN, Morin PA (2004) A novel method for collection and preservation of faeces for genetic studies. Mol Ecol Notes 4:761–764

Rudnick JA, Katzner TE, Bragin EA, Rhodes EJR, Dewoody JA (2005) Using naturally shed feathers for individual identification, genetic parentage analyses, and population monitoring in an endangered Eastern Imperial Eagle (*Aquila heliaca*) population from Kazakhstan. Mol Ecol 14:2959–2967

Rudnick JA, Katzner TE, Bragin EA, DeWoody AD (2007) Species identification of birds through genetic analysis of naturally shed feathers. Mol Ecol Notes 7:757–762

Seki S (2006) Application of molted feathers as noninvasive samples to studies on the genetic structure of pigeons (Aves: Columbidae). J For Res Jpn 11:125–129

Solberg KH, Bellemain E, Drageset O-M, Taberlet P, Swenson JE (2006) An evaluation of field and non-invasive genetic methods to estimate brown bear (*Ursus arctos*) population size. Biol Conserv 128:158–168

Stoneking M (1995) Ancient DNA: how do you know when you have it and what can you do with it? Am J Hum Genet 57:1259–1262

Strasser H, Weber C (1999) On the asymptotic theory of permutation statistics. Math Methods Stat 8:220–250

Taberlet P, Bouvet J (1991) A single plucked feather as a source of DNA for bird genetic studies. Auk 108:959–960

Taberlet P, Luikart G (1999) Non-invasive genetic sampling and individual identification. Biol J Linn Soc 68:41–55

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. Trends Ecol Evol 14:323–327

R Development Core Team (2011) R: A Language and Environment for Statistical Computing R Development Core Team R Foundation for Statistical Computing, Vienna, Austria, 2011, ISBN 3-900051-07-0 http://www.R-project.org

Teare JM, Islam R, Flanagan R, Gallagher S, Davies MG, Grabau C (1997) Measurement of nucleic acid concentrations using the DyNA Quant and the GeneQuant. Biotechniques 22:1170–1174

Väli Ü, Dombrovski V, Treinys R, Bergmanis U, Daróczi SzJ, Dravecky M, Ivanovski V, Lontkowski J, Maciorowski G, Meyburg B, Mizera T, Zeitz R, Ellegren H (2010) Widespread hybridization between the Greater Spotted Eagle *Aquila clanga* and the Lesser Spotted Eagle *Aquila pomarina* (Aves: Accipitriformes) in Europe. Biol J Linn Soc 100:725–736

Waits LP, Patkeau D (2005) New non-invasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. J Wildl Manag 69:1419–1433

Wasser SK, Houston CS, Koehler GM, Cadd GG, Fain SR (1997) Techniques for application of feacal DNA methods to field studies of Ursids. Mol Ecol 6:1091–1097

Weigmann H-D (1968) Reduction of disulfide bonds in keratin with 1,4-dithiothreitol. I. Kinetic investigation. J Polym Sci A-1 6:237–2253

Weiß BM, Poggemann K, Olek K, Foerester K, Hirschenhauser K (2008) Isolation and characterization of microsatellite marker loci in the greylag goose (*Anser anser*). Mol Ecol Resour 8:1411–1413

Zayats T, Mackey DA, Malecaze F, Calvas P, Guggenheim JA (2009) Quality of DNA extracted from mouthwashes. PLoS ONE 4:e6165