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TECHNICAL ADVANCES

DNA sampling from eggshell swabbing is widely applicable in wild bird populations as demonstrated in 23 species

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Abstract

There is increasing interest in noninvasive DNA sampling techniques. In birds, there are several methods proposed for sampling DNA, and of these, the use of eggshell swabbing is potentially applicable to a wide range of species. We estimated the effectiveness of this method in the wild by sampling the eggs of 23 bird species. Sampling of eggs was performed twice per nest, soon after the clutch was laid and again at the end of egg incubation. We genotyped DNA samples using a set of five conserved microsatellite markers, which included a Z-linked locus and a sex-typing marker. We successfully collected avian DNA from the eggs of all species tested and from 88.48% of the samples. In most of the cases, the DNA concentration was low (ca. 10 ng/μL). The number of microsatellite loci amplified per sample (0–5) was used as a measure of the genotyping success of the sample. On average, we genotyped 3.01 ± 0.12 loci per sample (mean ± SE), and time of sampling did not seem to have an effect; however, genotyping success differed among species and was greater in those species that used feather material for lining their nest cups. We also checked for the occurrence of possible genotyping errors derived from using samples with very low DNA quantities (i.e. allelic dropout or false alleles) and for DNA contamination from individuals other than the mother, which appeared at a moderate rate (in 44% of the PCR replicates and in 17.36% of samples, respectively). Additionally, we investigated whether the DNA on eggshells corresponded to maternal DNA by comparing the genotypes obtained from the eggshells to those obtained from blood samples of all the nestlings for six nests of magpies. In five of the six magpie nests, we found evidence that the swab genotypes were a mixture of genotypes from both parents and this finding was independent of the time of incubation. Thus, our results broadly confirm that the swabbing of eggshells can be used as a noninvasive method for obtaining DNA and is applicable across a wide range of bird species. Nonetheless, genotyping errors should be properly estimated for each species by using a suite of highly polymorphic loci. These errors may be resolved by sampling only recently laid eggs (to avoid non-maternal DNA contamination) or by performing several PCR replicates per sample (to avoid allelic dropout and false alleles) and/or by increasing the amount of DNA used in the PCR through increasing the volume of the PCR or increasing the concentration of template DNA.

Keywords: Aves, DNA sampling, egg surface, eggshells, maternal DNA, noninvasive sampling

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Introduction

Studies in molecular genetics are having a great impact on our knowledge regarding the ecology and evolution of animals. Molecular genetics provides us with important information about the phylogenetic relationships and systematics of organisms, population genetics, mating systems and micro-evolutionary processes (e.g. Griffith *et al*. 2002; Avise 2004; Hackett *et al*. 2008). The sampling of genetic material is therefore a necessary step for many studies trying to answer evolutionary and/or ecological questions. In birds, the preferred source of DNA is blood because it contains nucleated red blood cells with abundant DNA; however, other materials such as plucked feathers (Taberlet & Bouvet 1991; Bello *et al*. 2001; Harvey *et al*. 2006) or buccal swabs (Handel *et al*.

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2006) can also be used for collecting DNA. All these methods, however, require the capture and manipulation of individuals, which disturbs the animals under study, can sometimes be challenging and requires significant effort by researchers (reviewed in Schmaltz *et al.* 2006). For birds, it is known that handling individuals can provoke an increased level of stress (Mueller *et al.* 2006) and if performed during the breeding season might result in unusual behaviour and/or in nest desertion (Criscuolo 2001). Moreover, taking blood samples may reduce annual survival rates (Brown & Brown 2009; but see Sheldon *et al.* 2008). Therefore, there are situations when the capturing and handling of individuals are not recommended, such as for those belonging to endangered populations or when capturing dramatically disturbs the animals under study. In these cases, the use of a noninvasive DNA sampling is the only possible or ethical approach (Taberlet *et al.* 1999).

During the two last decades, many scientific articles have described or improved techniques for DNA collection that do not require the handling of animals (reviewed in Taberlet *et al.* 1999; Piggott & Taylor 2003; Waits & Paetkau 2005). In birds, DNA can be collected from diverse resources (see Table 1 for a review); however, all of these methods do not have the same potential for being classified as a noninvasive method for avian DNA collection across a wide range of species. For instance, DNA sampling from lining feathers can only be performed in some bird species that use their *own* feathers as lining material (see e.g. Snow *et al.* 1998; Hansell 2000). The feather-trap described by Maurer *et al.* (2010) might not be effective for more suspicious bird species such as black-billed magpies (*Pica pica*), who will not enter their nests when they detect unusual material within the nest (DM-G pers. obs.). Sampling hatched eggshells would be difficult in species where adults remove them from the nest soon after hatching (Tinbergen 1963; Derrickson & Warkentin 1991). Collecting powder from eggshells would be difficult to perform and risky in species with thin eggshells and small eggs.

The use of swab samples from the external egg surface proposed by Schmaltz *et al.* (2006) still involves human nest disturbance, although it does not have the problems mentioned in the previous paragraph and we think it has a high potential to be effective in a wide range of bird species. This method relies on collecting some of the mother's cells from the reproductive tract or skin of incubating individuals that adhered to the eggshell and without physically capturing the target individuals (Schmaltz *et al.* 2006). Schmaltz *et al.* (2006) successfully collected maternal DNA from 86 to 90% of the sampled nests in the field of herring gull (*Larus argentatus*) and Caspian tern (*Sterna caspia*), indicating this method may be of utility in many studies. Despite the potential advantages of this method, as far as we know, it has never been used in any other bird species. These promising results could be biased by the small number of species on which the method has been tested ($n = 2$) as well as the possibility that only recently laid eggs, and mainly those with blood remains on the eggshells (which are common in these two species, see Schmaltz *et al.* 2006), were required for collecting DNA. Information on the applicability of this method to a variety of phylogenetically distant bird species, differing in size or life history traits, as well as an estimation of the effectiveness of the methods depending on the sampling time (i.e. days after egg laying) is desirable for researchers intending to adopt this noninvasive technique for collecting maternal DNA.

In this article, we try to fill this gap and assess the applicability of swabbing eggshells to collect the DNA of adults in multiple bird species and to determine whether its success is dependent on the time of egg incubation.

Table 1 Noninvasive techniques used to collect DNA in birds

Material	Citation
Feathers	
Moulted or used as lining material	Pearce *et al.* 1997; Bush *et al.* 2005; Segelbacher 2002; Gebhardt *et al.* 2009; Miño & Del Lama 2009
Plucked by a ''feather-trap''	Maurer *et al.* 2010
Excrements	
Faeces	Robertson *et al.* 1999; Idaghdour *et al.* 2003; Regnaut *et al.* 2006; Guerrini & Barbanera 2009; Marrero *et al.* 2009
Urine	Nota & Takenaka 1999
Food	
Regurgitated seeds	Marrero *et al.* 2009
Eggshells	
From hatched or predated eggs	Bush *et al.* 2005; Trimbos *et al.* 2009
By filing a small piece of the external eggshell	Egloff *et al.* 2009
By swabbing the egg surface	Schmaltz *et al.* 2006

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Schmaltz *et al*. (2006) sampled the eggs soon after laying (within 48 h) and used a single swab of each egg. However, we swabbed the entire clutch of eggs with a single swab (2–10 eggs) during incubation to maximize the probability of collecting bird DNA, although this strategy increases the chance of amplifying DNA from the male or other individuals that might visit the nest. We sampled 141 nests belonging to 23 bird species (9 non-passerines and 14 passerines) and assessed the source of the DNA using four microsatellite loci, including a Z-linked locus and a sex-typing marker. The loci used displayed a high utility across a wide range of species. Universal microsatellite primer sets suitable for genotyping most species of birds are now available (Dawson *et al*. 2010) and therefore we can check our swabbing methodology in multiple species and compare the results. Evidence of null alleles (Pemberton *et al*. 1995) and genotyping errors, such as allelic dropout (i.e. when one allele of a heterozygous individual is not detected because of the PCR, Taberlet *et al*. 1996) and false alleles (i.e. PCR-generated artefacts, Taberlet *et al*. 1996), were also checked in those species in which the loci used were polymorphic. To examine whether DNA recovered from the shell exterior was exclusively from the mother, we also compared nestling genotypes from six magpie nests to the swab sample. Lastly, we performed a comparative analysis to attempt to detect which species-specific life history traits (nest materials used, type of nest (open or closed), the start of full incubation, egg size and clutch size) are positively related to the probability of successful DNA isolation from eggshell swabs. Results from this comparative analysis would be useful if we could detect any association that can be used as an indicator of the likely utility of the method in a target bird species or population.

Materials and methods

Field procedures

We sampled eggshells in 4–8 nests per species during the 2007–2008 breeding seasons (see Table 2 for species list and sample sizes). These species were breeding in natural nests or nest boxes in the south of Spain, most of them in Hoya of Guadix (37°14′ N, 3°11′ W). We used samples of the following species breeding in other Spanish localities:

Table 2 Clutch sizes, number of nests sampled (N) and number of samples successfully amplified for each marker in each species during the beginning of egg incubation (*initial samples*) and at the end of egg incubation (*final samples*). In all cases, the numbers before slashes refer to number of the *initial samples* amplified and the number after corresponds to the number of *final samples* amplified

Species	Latin name	Clutch size median (min–max)	*N* (initial/final)	TG04–004	TG07–022	TG12–015	Z-002A	Z-054
Great tit	*Parus major*	10 (9–10)	4/5	4/4	3/5	3/4	4/4	3/4
Barn swallow	*Hirundo rustica*	5 (4–5)	5/5	5/4	5/1	5/5	5/5	4/3
Spotless starling	*Sturnus unicolor*	4 (3–6)	5/4	5/3	5/3	5/2	5/2	5/3
Eurasian blackbird	*Turdus merula*	3 (2–4)	6/6	3/2	1/1	0/1	5/2	2/2
Black wheatear	*Oenanthe leucura*	4 (3–5)	6/5	4/3	2/3	5/4	3/4	2/2
House sparrow	*Passer domesticus*	5 (4–6)	6/6	6/3	4/2	6/3	5/4	6/1
Tree sparrow	*Passer montanus*	5 (5–6)	6/2	5/1	3/0	5/0	4/0	3/0
Rock sparrow	*Petronia petronia*	7 (5–8)*	4/5	4/1	2/0	3/2	3/2	1/2
European serin	*Serinus serinus*	4 (3–4)	6/1	4/1	3/1	2/1	5/1	1/0
Southern grey shrike	*Lanius meridionalis*	6 (5–6)	6/5	5/5	2/4	3/4	4/5	4/4
Jackdaw	*Corvus monedula*	5.5 (3–7)	6/6	4/5	2/1	3/3	4/6	2/3
Carrion crow	*Corvus corone*	5 (3–5)	6/3	6/2	6/1	4/2	6/2	4/2
Black-billed magpie	*Pica pica*	6 (5–8)	8/8	8/7	8/8	8/7	8/8	8/8
Red-billed chough	*Pyrrhocorax pyrrhocorax*	5 (4–5)	6/3	3/3	2/1	2/3	3/2	3/3
Hoopoe	*Upupa eppops*	8 (6–9)	5/6	2/4	3/4	0/3	2/2	0/1
European roller	*Coracias garrulus*	6 (6–7)	4/4	3/1	0/0	2/0	2/1	1/0
Pallid swift	*Apus pallidus*	2 (2–3)	5/3	5/3	2/1	3/2	3/3	3/1
Rock dove	*Columba livia*	2 (2–2)	5/3	5/1	4/1	5/2	4/1	1/1
Woodpigeon	*Columba palumbus*	2 (2–2)	6/—†	6/—†	4/—†	6/—†	5/—†	1/—†
Common kestrel	*Falco tinunculus*	5 (4–5)	5/3	3/1	0/0	1/0	2/0	2/0
Long-eared owl	*Asio otus*	4 (4–5)	4/1	4/1	3/1	4/1	4/1	4/1
Scops owl	*Otus scops*	4 (3–5)	6/5	5/2	2/1	3/2	3/4	3/1
Little owl	*Athene noctua*	5 (4–5)	4/4	3/2	0/0	0/0	2/2	0/1

*One rock sparrow nest contained 14 eggs at the final sampling, probably from two females. It was excluded from the estimations of clutch sizes.
†No *final samples* were obtained at the end of incubation for woodpigeon because all nests found were depredated during incubation.

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Eurasian blackbird (*Turdus merula*), pallid swift (*Apus pallidus*) and European serin (*Serinus serinus*) – these samples were collected within the city of Granada (37°11′N, 3°36′W); black-billed magpie, whose samples came from two populations in the National Park of Doñana (36°59′ N 6°26′W) and close to Iznalloz town (37°25′N 3°33′W); and house sparrow (*Passer domesticus*), which were sampled close to the city of Badajoz (38°52′N 6°58′W).

The eggs of each clutch were swabbed on two occasions: 1–2 days after completion of laying (hereafter *initial samples*) and 2–4 days before hatching (hereafter *final samples*). However, for some nests, we were only able to collect one sample because either the nests were found a few days before hatching, or the nests were depredated or abandoned after initial sampling (Table 2). Additionally, in magpie nests, we performed an additional third sampling during the laying period (0–5 days after the first egg was laid; i.e. two eggs were laid within 48 h). For sampling each clutch, we wore new latex gloves sterilized with 96% ethanol to prevent inter-nest contamination and to keep conditions as aseptic as possible. Each pair of new gloves was only used for one clutch and then discarded. Once the ethanol had evaporated from gloves, we gently handled and sampled eggshells by rubbing the whole eggshell with a sterile swab (EUROTUBO®, Delta-Lab) which we had moistened with sterile sodium phosphate buffer (0.2 M; pH = 7.2). All the eggs in the nest were sampled with the same swab by wiping the complete egg surface. Afterwards, the swab was placed in a rubber-sealed microfuge tube with 600 μl of sterile sodium phosphate buffer (0.2 M; pH = 7.2) (except for magpies where we used 1.2 mL of buffer). Samples were transported in a portable refrigerator at 4–6 °C to the laboratory where they were kept frozen at −20 °C until the DNA was extracted. During sampling, we noted the number of sampled eggs as well as the width and the length of each egg (measured using a digital calliper, accurate to ±0.01 mm). We did not sample any eggs showing traces of damage or breakage to avoid the risk of possible contamination of the egg swab sample by the DNA from nestlings. Contrary to what was found in the two species (a gull and tern) tested by Schmaltz *et al.* (2006), we found that, independent of the species, blood smears on the egg surface were only apparent sporadically and were detected in <1% of cases (Peralta-Sánchez *et al.* unpublished data) and, consequently, we did not use this information in our analyses.

DNA isolation

We extracted DNA using a Chelex-based protocol recently optimized for eggshell swab samples and used for the identification of microbial communities of avian eggshell surfaces (Martín-Platero *et al.* 2010). Briefly, the swab was placed into a sterile 1.5-mL microfuge tube and centrifuged in a microfuge for 5 min at 20 000 *g* to extract any cells adhering to the swab. Afterwards, the swab was discarded and the filtrate was returned to the original tube with the remaining sample. The sample was again centrifuged for 5 min at 20 000 *g*, and then the supernatant was discarded. The pellet was suspended in 100 μL of 0.1 × TE buffer (1 mM Tris, 0.1 mM EDTA) with 1 mg of lysozyme and incubated for 45 min at 37 °C. After this period, 1 μL of 10 mg/mL proteinase K and 1 μL of 10% sodium dodecyl sulphate (SDS) were added and incubated for a further 30 min at 37 °C. Then, 100 μL of 10% *Chelex-100* (200–400 mesh, Bio-Rad) was added, gently mixed and incubated for 30 min at 56 °C. Subsequently, samples were vortexed for 10 s and incubated for 10 min at 100 °C. Finally, samples were centrifuged for 5 min at 20 000 *g*, and the supernatant was transferred to a new microfuge tube (for more details about this protocol, see Martín-Platero *et al.* 2010). DNA concentration was assessed by fluorimetry (Fluostar Optima, BMG Labtech Ltd.) and using a set of known concentration DNA standards isolated from calf thymus (6.25, 12.5, 25, 50 and 100 ng/μL). We also measured 79 blank samples jointly with the swab samples. Blank samples were used to calculate the detection limit of our fluorometer, which was defined as three times the standard deviation of values of blank samples (see MacDougall *et al.* 1980).

DNA extractions were performed in the Laboratory of Microbiology at the University of Granada (Spain). This laboratory has not been used for PCRs with DNA samples from birds; thus, we avoided any possible PCR-generated contamination during our DNA extractions.

Genotyping

The genotyping of our DNA samples was performed in the Molecular Ecology Laboratory at the University of Sheffield (England). We used conserved primer sets for four microsatellite loci with a high utility across a wide range of bird species and a sex-typing marker. In short, we used three autosomal microsatellite loci: *TG04-004*, *TG07-022* and *TG12-015* (Dawson *et al.* 2010), a Z-linked microsatellite locus (*Z-054*, Dawson *et al.* unpublished data) and a sex-typing marker (*Z-002A*, Dawson 2007). Birds have a ZW sex determination system; locus Z-002 is located on the Z and W chromosomes with alleles of different in size, and hence this locus can be used to distinguish between male and female nonratite birds (see Dawson 2007 for more details). Female birds always display only one Z-allele when amplified with a Z-linked locus (i.e. they are always homozygous (hemizygous)), whereas males possess two Z-alleles so they can be homozygous or heterozygous in those species in which

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this locus is polymorphic. Locus *Z-054* was selected because the presence of a heterozygote at this locus would indicate the presence of DNA from an individual other than the mother (male DNA or DNA from more than one female). This locus has also previously been found to be Z-linked and polymorphic in several bird species [zebra finch (*Taeniopygia guttata*), A. Ball pers. comm.; gouldian finch (*Erythrura gouldiae*), K.-W. Kim pers. comm.; and in Seychelles warbler (*Acrocephalus sechellensis*) and chicken (*Gallus gallus*), D.A. Dawson pers. comm.] and has been found to amplify in a wide range of bird species (D.A. Dawson *et al.* unpublished data) as has occurred in other markers designed in a similar way. The primer sets for the autosomal loci and sex-typing marker were designed from a consensus sequence of conserved zebra finch expressed sequence tag (EST) microsatellite sequences and aligned to their chicken homologues (Dawson 2007; Dawson *et al.* 2010). The primer set for the Z-linked locus was designed similarly but using a microsatellite sequence (*Ase50* locus, Richardson *et al.* 2000) of Seychelles warbler and aligned to its chicken homologue (*Z-054*, Dawson *et al.* unpublished data, forward primer sequence: CTGTCTGGCATGCTGACTC, reverse primer sequence: ATCAGCAGACAACATGGACTC). We chose the three autosomal loci from the 33 autosomal loci available from Dawson *et al.* (2010) according to their potential polymorphism (estimated as the proportion of species that were polymorphic, Dawson *et al.* 2010). These four loci were combined into a single multiplex PCR with the sex-typing marker *Z-002A*. We used MULTIPLEX MANAGER software (Holleley & Geerts 2009) to test the different primer sets combinations according to their product sizes, colour of fluorescent labels and possible incompatibilities based on primer sequence homology. In this way, we could genotype DNA samples belonging to different bird species in a unique single PCR containing all five primer sets.

The dilution of DNA samples can help to eliminate PCR inhibitors that may occur in samples that are not well purified (see e.g. Martín-Platero *et al.* 2010). We performed several preliminary assays with different dilutions (1:1–1:100 dilutions) of eight (magpie) DNA samples to estimate the optimal dilution of samples required. Dilutions of 1:10 and 1:50 of DNA samples gave the highest number of positive amplifications (results not shown). Accordingly, we performed two PCRs on the same DNA samples, one diluted ten-fold and the other fifty-fold (thereafter PCR-10 and PCR-50, respectively). For each PCR, we used 1 μL of diluted DNA sample (1:10 or 1:50). Once DNA samples were placed into the 96-well microtitre plate, we incubated them for 10 min at 37 °C to evaporate the water. Afterwards, we added the PCR reagents. Each sample was PCR amplified in a 4-μl reaction volume, containing 2 μL of QIAGEN Multiplex PCR Kit (Qiagen) that includes HotStartTaq DNA polymerase, dNTP mix and PCR buffer (6 mM $MgCl_2$, Factor MP and unknown additives), and 2 μL of low TE (10 mM Tris, 0.1 mM EDTA) buffer containing 0.2 μM of each primer. We included several negative (11 to 33) and two positive controls per 96-well PCR plate. Negative controls had all the PCR constituents in the same volume but without the DNA sample. The positive control contained ca. 15 ng of blood-extracted magpie DNA. PCR amplifications were performed in a DNA Engine Tetrad PTC-225 Peltier thermal cycler (MJ Research). The PCR profile used was 95 °C for 15 min, then 35 cycles of 94 °C for 30 s, 58 °C for 90 s, 72 °C for 60 s, followed by one cycle of 30 min at 60 °C. PCR products were separated on an ABI 3730 DNA Analyser, and allele sizes were scored using GENEMAPPER software v3.7 (Applied Biosystems). We used the genotypes obtained from the PCR-10 and PCR-50 dilutions to make a table of the combined genotypes. The combined genotype was made by considering all alleles amplified in any of two PCR replicates for each sample and locus (see Table S1). Genotypes were checked for any evidence of errors by comparing the observed allele sizes with those sizes expected based on the (cloned) origin sequence and by comparing the genotypes obtained in both PCRs. Nonetheless, it should be noted here that the detected rates of genotyping error are underestimated because some of these markers are monomorphic in some of the bird species tested or, even for those loci that were polymorphic, the number of alleles per locus was not particularly high (Fig. 1), so we cannot be sure that we are detecting all genotyping errors. Similarly, we could only detect a possible contamination from the male in those species where *Z-054* was polymorphic (Fig. 1). Moreover, the level of polymorphism of the markers used decreases as the genetic distance between the target species and the source species of the locus (zebra finch) increases (see Dawson *et al.* 2010); therefore, our estimates of genotyping errors would be especially underestimated in species genetically distant to zebra finch.

The number of microsatellite loci successfully genotyped per sample (0–5) was used as a measure of genotyping success. We calculated genotyping success by using the genotypes obtained from both the PCR-10 and PCR-50 data and from the combined genotypes resulting from comparison of both PCR (thereafter combined-genotyping success). To estimate whether the correct target locus was being amplified, the expected allele sizes based on the zebra finch sequence were compared to the allele sizes observed from the egg swab samples: 166 bp for *TG04-004*, 283 bp for *TG12-015* and 416 bp for *TG07-022* (Dawson *et al.* 2010); 254 bp for *Z-054* (Dawson *et al.* unpublished data); and 209 and 219 bp for *Z-002A* (sizes of alleles on W and Z

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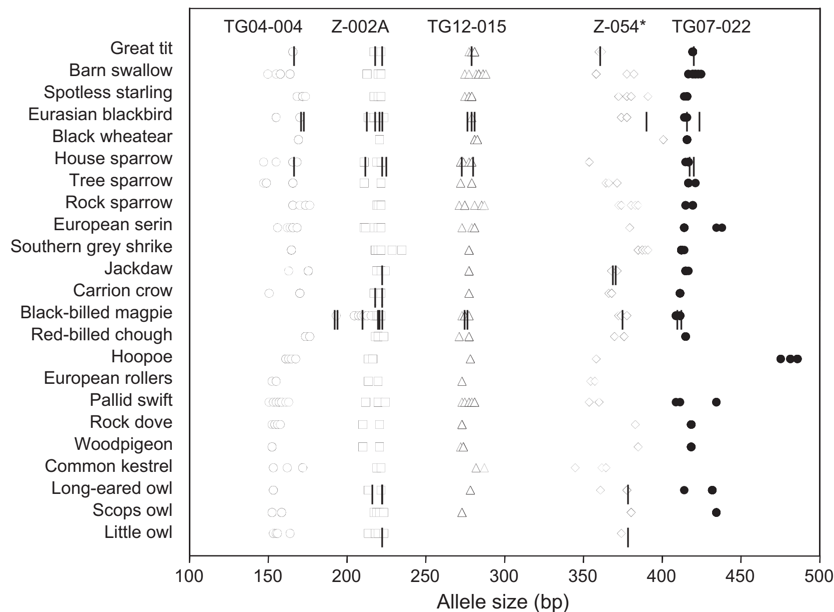

Fig. 1 Observed allele sizes (bp) for five microsatellite markers amplified in 23 species of birds. Vertical bars indicate the allele sizes observed for that marker in previous studies and when amplified using DNA isolated from blood samples. *We added 100 bp to all allele sizes of the *Z-054* locus to avoid the allele sizes overlapping with those of locus *TG12-015* in the figure.

chromosomes, respectively, Dawson 2007). When available, we compared the observed allele sizes obtained in previous studies with the observed allele sizes from the egg swab samples. Comparison was possible with allele sizes of the three autosomal loci when amplified in four passerine species (black-billed magpie, Eurasian blackbird, great tit and house sparrow, Dawson *et al.* 2010), and the *Z-054* locus and the *Z-002A* sex-typing marker amplified in seven species (black-billed magpie, carrion crow, Eurasian blackbird, great tit, house sparrow, little owl, long-eared owl, Dawson 2007; D.A. Dawson *et al.* unpublished data).

Comparison between nestling and egg swab genotypes in magpies

We tried to collect a feather sample from some female magpies by using a feather-trap similar to that described by Maurer *et al.* (2010). However, we were not successful because magpie adults did not enter their nests while the trap was set, most probably because they detected unusual materials within the nest. Capture of magpie adults in our population, although possible, is a difficult task and we have observed that it can provoke nest desertion if performed during the breeding season (M. Molina-Morales & J. G. Martínez, pers. com.). For this reason, we compared the genotypes obtained from egg swab samples and those obtained from blood samples of all hatched nestlings in six magpie nests to attempt to validate the assumption that avian DNA isolated from eggshell corresponded DNA of one parent (presumably the female, as found by Schmaltz *et al.* (2006)). Genomic DNA from nestlings was extracted using an ammonium acetate precipitation method (Nicholls *et al.* 2000) from blood samples collected soon after hatching *via* brachial vein puncture. We chose seven highly polymorphic loci for black-billed magpies that did not display any significant deviations from Hardy–Weinberg equilibrium and whose estimated null allele frequencies were <0.05 (Martín-Gálvez *et al.* 2006, 2009 and unpublished data), namely: *Ase18* and *Ase64* (Richardson *et al.* 2000), *PmaTGAn42* (Saladin *et al.* 2003), *Ppi2* (Martínez *et al.* 1999), *Ppi008* and *Ppi012* (Martín-Gálvez *et al.* 2009) and *TG13-017* (Dawson *et al.* 2010). PCRs were performed similarly to those previously described earlier for the egg swab samples with the following modifications: the reaction volume was 2 μL instead of 4 μL, only one dilution of DNA sample was PCR amplified (1:10), and for the PCR annealing temperature, we used 57 °C for *Ase64* and 59 °C for *PmaTGAn42*, *Ppi2*, *Ppi008* and *Ppi012*.

Statistical analyses

Variables were graphically checked for normal distribution of their frequencies (density and normal probability plots) and, if necessary, non-parametric statistical tests were used. A median test was used to analyse the effect of DNA dilution on genotyping success (differences between the PCR-10 and PCR-50 data) and variation

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among species in combined-genotyping success. We used a *t*-test for dependent samples to compare combined-genotyping success between *initial* and *final samples*. Comparisons of genotyping success obtained from the PCR-10 test, the PCR-50 test and combined genotypes were analysed by using Wilcoxon matched pairs tests. Friedman ANOVA and Kendall's coefficient of concordance were used to analyse variation of genotyping success per locus among species. All these analyses were performed in STATISTICA v8.0 software (StatSoft Inc. 2008). All tests were two-tailed, and the values provided are means ± SE.

For analysing interspecific differences in genotyping success in relation to several life history traits of the species sampled, we performed phylogenetically controlled generalized linear models (PGLS). We obtained from Snow *et al.* (1998) information for each bird species relative to (i) type of nest (open nest or hole nest) (except for the common kestrel whose nests were located on magpie old nests, i.e. open nests); (ii) sex responsible for incubation (i.e. if only by the female or if helped by the male); (iii) if nestlings hatch synchronously or not, as an estimate of time between laying of the first egg and the start of full incubation; and (iv) the use of nest-lining material (except for woodpigeon that does not use this material in our study area, DM-G pers. obs.). As further predictors of genotyping success, we also calculated from our samples the averages per species of (v) clutch size and (vi) egg surface area sampled per clutch. We used combined-genotyping success as the dependent variable. We performed these analyses separately for the *initial* and the *final samples* because differences about what variables influence genotyping success can be predicted between both periods. These analyses were performed within phylogenetic generalized least squares (PGLS) using R software v2.9.2 (R Development Core Team 2009) and APE (R package, v 2.4-1, Paradis *et al.* 2004) with an additional unpublished function by R. Freckleton (pglm3.3R.r; available from R. Freckleton on request), which allows the inclusion of discrete variables as independent factors. We considered the phylogeny of species according to that described by Sibley & Ahlquist (1990) and shown in Fig. 2. The PGLS approach controls the phylogenetic relationships among species by incorporating a matrix of the covariances (Martins & Hansen 1997; Pagel 1999). The method applies likelihood ratio statistics to test hypotheses of correlated trait evolution and to estimate the importance of phylogenetic corrections in the models (Freckleton *et al.* 2002). The degree of phylogenetic dependence (λ, with values between 0 and 1) was estimated for each model. Values of λ significantly <1 would indicate traits that are less similar among species than expected from their phylogenetic relationship, whereas the reverse is suggested when λ = 1. We tested the 63 possible models resulting from combinations of all predictor variables but excluding interaction factors. Afterwards, we used values of the second-order information criterion for finite samples (AICc, Sugiura 1978) to calculate the Akaike weights and the evidence ratios according to Burnham & Anderson (2002).

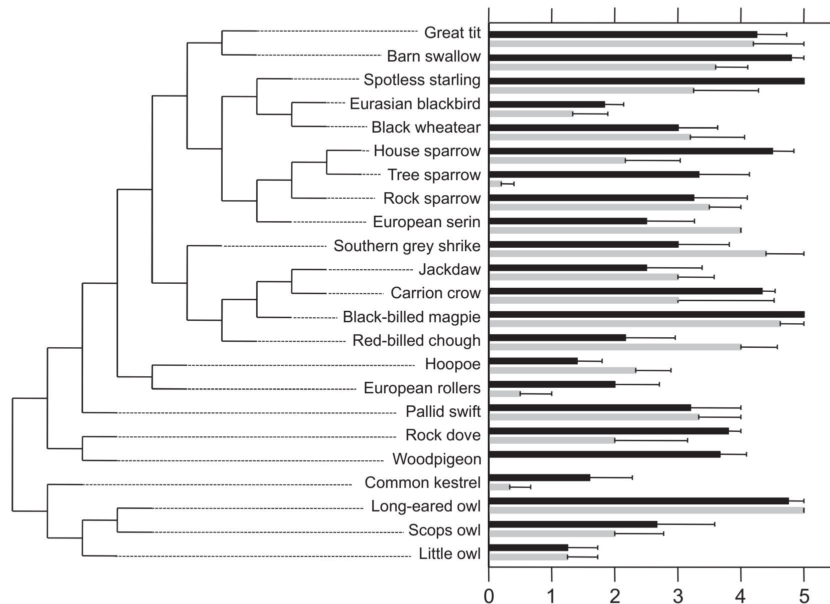

Fig. 2 Mean ± SE of the number of markers amplified (0–5) per sample (combined-genotyping success) from DNA extracted from egg swab samples taken at the beginning of egg incubation (*initial samples*, black bars) and at the end of egg incubation (*final samples*, grey bars) in each of 23 bird species. The phylogeny of the bird species tested according to Sibley & Ahlquist (1990) is also shown.

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Results

Genomic DNA concentrations of the egg swab samples

Genomic DNA concentrations were up to a maximum of 87.08 ng/μL (mean ± SE: 8.38 ± 0.61 ng/μL) and the volume obtained was ca. 150 μL. On average per species, the DNA concentration of *initial samples* was 8.98 ± 1.15 ng/μL (N = 23 species, 123 samples) and 7.23 ± 0.79 ng/μL (N = 22 species, 93 samples) for *final samples*. Nonetheless, the detected DNA concentrations were very low, and most of them (96.3%, N = 216) were below the detection limit of our fluorometer (21.78 ng/μL). Therefore, we did not use the DNA concentration obtained from swab samples in further statistical analyses.

Genotyping success

We obtained the combined genotype for at least one of the five microsatellite loci used in 114 of the 123 *initial samples* from 23 bird species (92.68%) and in 76 of the 93 of *final samples* from 22 bird species (81.72%) (see combined genotypes for all samples in the Table S1). The number of samples successfully genotyped differed among loci used (Table 2, Friedman ANOVA; *initial samples*: $N = 23$, df = 4, $\chi^2 = 39.71$, $P < 0.0001$; *final samples*, $N = 22$, df = 4, $\chi^2 = 18.07$, $P = 0.001$), although these differences were partially (*initial samples*) or slightly (*final samples*) correlated among species (Kendall's coefficient of concordance were 0.43 and 0.21, respectively). Only two loci completely failed to amplify in all the samples of a particular species. Marker *TG12-015* completely failed to amplify for all individuals sampled from one species (little owl), and *TG07-022* failed to amplify in all individuals for three species (little owl, common kestrel and European rollers; Table 2 and Fig. 1).

In relation with the number of markers successfully genotyped per sample (i.e. values from 0 to 5), 3.01 ± 0.12 (mean ± SE) loci were genotyped per sample. We found PCR-10 gave a significantly greater genotyping success than that obtained from PCR-50 and this occurred in both the *initial samples*: DNA dilution effect: 2.81 ± 0.17 vs. 2.14 ± 0.16 (Wilcoxon matched pairs test: 123 samples, 23 species, $Z = 4.64$, $P < 0.0001$), and in the *final samples* (2.39 ± 0.20 vs 1.68 ± 0.17; Wilcoxon matched pairs test: 93 samples, 22 species, $Z = 4.59$, $P < 0.0001$). However, we detected a considerable interspecific variation in the effect of DNA dilution on the number of markers successfully genotyped (median tests with differences in genotyping success between PCR-10 and PCR-50 as the response variable and bird species as a grouping variable; *initial samples*: $\chi^2 = 30.89$, df = 22, $P = 0.098$; *final samples*: $\chi^2 = 41.48$, df = 22, $P = 0.007$). Interestingly, when using the combined-genotyping success (see Materials and Methods), values were significantly higher than those obtained from PCR-10 or PCR-50 (*initial samples*: combined-genotyping success 3.24 ± 0.16, Wilcoxon matched pairs test: 123 samples from 23 species, combined vs. PCR-10 ($Z = 5.37$, $P < 0.0001$) and combined vs. PCR-50 ($Z = 7.57$, $P < 0.0001$); *final samples*: combined-genotyping success: 2.71 ± 0.20; Wilcoxon matched pairs tests: 93 samples, combined vs. PCR-10 ($Z = 4.20$, $P < 0.0001$) and combined vs. PCR-50 ($Z = 6.51$, $P < 0.0001$). These results are a consequence of some genotypes obtained from PCR-10 failing to amplify in the PCR-50 reaction and *vice versa* (see Table S2). We did not find differences for combined-genotyping success between the *initial* and *final samples* (3.19 ± 0.26 vs. 2.78 ± 0.30; t-test for dependent samples (mean per species): $t = 1.48$, df = 21, $P = 0.16$), although combined-genotyping success differed among bird species (Fig. 2, median tests: *initial samples*, $N = 123$, $\chi^2 = 53.51$, df = 22 $P = 0.0002$; *final samples*: $\chi^2 = 36.53$, df = 22, $P = 0.027$).

Allele sizes for the five loci in the different species tested

The observed allele sizes in most of the species were very similar to the expected sizes based on zebra finch sequences (Fig. 1). Allele sizes for *TG04-004* locus in magpies and *TG07-022* locus in hoopoes had the largest differences with respect to their expected sizes (Fig. 1). Concerning the magpies, the alleles sizes obtained from the egg swabs coincided with those obtained in the positive control (see Table S1) and from those reported in previous studies (Fig. 1 and Martín-Gálvez *et al.* 2009; Dawson *et al.* 2010; D.A. Dawson & G.J. Horsburgh unpublished data). Additionally, when information on allele sizes of loci studied were available from previous research studies, the sizes coincided with those observed in our samples (Fig. 1). This suggests that the correct target locus was being amplified in all species tested, with the possible exception of TG07-022 in the Hoopoes and confirmed the presence of avian DNA of the expected species on the egg surface of the studied species.

Comparison between the genotypes of magpie nestlings obtained from blood samples and those obtained from the swab of eggs belonging to the same clutch before hatching

In most of the cases, at least one allele at each locus was shared between nestlings and egg swab samples from the same nest, strongly suggesting that DNA on the egg surface belonged to one of their parents (possibly the female). However, in five of six magpie nests, we found evidence pointing to a possible mixture of genotypes of both parents (dagger in Table 3). Moreover, in two of

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Table 3 Genotypes obtained from the blood samples of hatched nestlings (n = 3–7) and three egg swab samples of the same six magpie nests. Magpie eggshells were sampled thrice: during laying (s0) and at the beginning (s1) and at the end (s2) of egg incubation. Number between parentheses indicates the number of nestlings with the stated genotype

Nest	Ase18	Ase64	PmaTGAn42	Ppi2	Ppi008	Ppi012	TG13-017
Z1B							
Nestlings n = 4	224/234 (2)	425/441 (2)	298/374 (2)	249/255 (3)	327/331 (2)	239/245 (1)	234/238 (1)
(blood samples)	234/234 (2)	429/433 (2)	326/374 (2)	249/257 (1)	329/331 (2)	239/251 (2)	236/238 (1)
						241/251 (1)	234/236 (2)
Egg swab s0	—/—	433/441	—/—	—*/257	327/329	245/251	—/—
Egg swab s1	—/—	—/—	—/—	—/—	—/—	—/—	232/234†
Egg swab s2	224/234	433/441	—/—	255/257	—/—	245/251	234/238†
Z3							
Nestlings n = 5	222/226 (1)	433/449 (3)	—/— (1)	245/275 (2)	—/— (1)	243/253 (1)	234/234 (5)
(blood samples)	222/230 (1)	449/453 (2)	278/286 (1)	255/275 (2)	327/327 (3)	245/253 (3)	
	224/226 (2)		278/374 (1)		327/331 (1)	245/259 (1)	
	224/230 (1)		282/286 (1)				
			282/374 (1)				
Egg swab s0	222/224	—/—	278/374†	—/—	—/—	—/—	234/234
Egg swab s1	222/224	433/453	286/374†	245/255	327/331	243/245	234/234
Egg swab s2	222/224	—/—	—/—	—/—	—/—	—/—	—/—
Z19							
Nestlings n = 7	222/—*(2)	429/— (1)	—/— (1)	245/245 (2)	—/— (1)	239/245 (1)	234/238 (3)
(blood samples)	222/242 (2)	429/449 (1)	270/286 (2)	245/255 (5)	327/331 (1)	239/247 (1)	232/238 (1)
	224/230 (1)	445/— (1)	270/310 (1)‡		331/331 (5)	245/251 (4)	234/— (2)
	230/234 (1)	445/449 (3)	278/286 (1)			245/255† (1)	232/236 (1)
	230/242 (1)	445/457 (1)	286/322 (2)				
Egg swab s0	222/230	—/—	278/286†	245/245	327/331	245/247	232/234/238†
Egg swab s1	—/—	—/—	—/—	—/—	—/—	—/—	—/—
Egg swab s2	—/—	—/—	—/—	—/—	—/—	—/—	—/—
Z30							
Nestlings n = 3	228/234 (3)	433/449 (2)	302/374 (3)	249/265 (1)	331/331 (3)	239/253 (1)	232/234 (1)
(blood samples)		445/449 (1)		265/— (1)		247/253 (2)	236/238 (2)
				265/271 (1)			
Egg swab s0	228/—*	433/—*	302/302	—*/265	331/331	—/—	—/—
Egg swab s1	—/—	—/—	302/302	249/265	—/—	239/247	234/238
Egg swab s2	218/228†	433/449	302/302	249/265	331/331	239/247/255	232/238
Z31							
Nestlings n = 7	228/234 (1)	429/433 (3)	—/— (2)	245/— (3)	—/— (3)	239/243 (4)	238/240 (7)
(blood samples)	228/242 (4)	429/457 (1)	286/302 (5)	245/249 (4)	327/329 (2)‡	239/253 (2)‡	
	234/242 (2)	433/445 (3)		327/331 (2)	241/243 (1)		
					329/333 (1)‡		
Egg swab s0	—/—	445/457†	302/302†	—/—	331/—*	243/—*	—/—
Egg swab s1	—/—	429/433†	286/286	245/245	—/—	243/—*	—/—
Egg swab s2	228/242	433/—*	—/—	—/—	—/—	—/—	—/—
Z41							
Nestlings n = 4	—/— (1)	—/— (2)	—/— (3)	—/— (1)	—/— (3)	—/— (1)	—/— (1)
(blood samples)	222/230 (2)	441/461 (2)	290/302 (2)	245/249 (1)	331/331 (2)	243/247 (2)	234/234 (4)
	230/234 (2)	449/461 (1)	249/251 (1)		247/253 (1)		
				251/271 (2)			
Egg swab s0	230/230	457/461	302/374	— */271	331/331	239/247	234/234
Egg swab s1	—/—	—/—	—/—	249/271	—/—	—/—	234/234
Egg swab s2	230/230	457/461	302/374	249/271	331/331	239/247	—/—

*Possible presence of allelic dropout following comparison of the genotype data obtained from the blood samples with all 3 egg swab samples.

†Possible mixture from both progenitor genotypes (identified by the presence of 3 or more alleles in at least one of the egg swab samples).

‡Nestling blood and egg swab genotypes did not share any alleles, which may be a result of allelic dropout (loci *Ppi008* and *Ppi012* in nest Z31), or could be because of a possible mixture of genotypes of both magpie parents combined with allelic dropout (locus *PmaTGAn42* in nest Z19).

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these nests (Z19 and Z31), one nestling for the *PmaTGAn42* locus, three nestlings for the *Ppi008* locus and two nestlings for *Ppi012* locus did not share any alleles with the swab samples (2.86% of the 210 nestling genotypes, i.e. 30 nestlings and 7 loci). These inconsistencies were possibly because of the presence of genotyping errors in the egg swab samples (see Table 3): allelic dropout (loci *Ppi008* and the *Ppi012* in nest Z31, Table 3) or a possible mixture of genotypes of both magpie parents and allelic dropout (locus *PmaTGAn42* in nest Z19, Table 3).

Genotyping errors detected in the egg swab samples

From 190 samples genotyped, we detected 18 samples belong to 10 species with more than two alleles in at least one of five loci suggesting more than one individual in a sample (green cells in Table S1). We also found 16 heterozygous individuals for the Z-linked locus *Z-054* in 10 species (i.e. suggesting the presence of DNA from an individual other than the mother, blue cells in Table S1). Furthermore, we also found one genotype in barn swallow, pallid swift and European serin (only in the final samples) slightly different to those obtained for the other egg swab samples in the same species (red cells in Table S1). The latter could indicate either the presence of false alleles or the contamination of DNA from other bird species (e.g. possibly caused by the use of feathers from other species as nest-lining material (Snow *et al.* 1998)). The frequency of these genotype errors did not differ between *initial samples* (21 of 116) and *final samples* (12 of 76, $\chi^2 = 0.17$, df = 1, $P = 0.68$). All errors in the genotypes obtained from the later egg swabs are probably due to the presence of DNA from more than one individual in these samples (probably from the male partner).

When comparing the results from PCR-10 and PCR-50, we found for 66 samples (for which their genotypes at least one locus did not match (44% of 88 possible comparisons) between the two PCR replicates of the same sample, see purple cells in the Table S2). This was mainly because of a certain individual possessing two alleles in one PCR, and only one of these alleles was amplified in the other PCR (i.e. allelic dropout or false alleles).

Specific factors determining genotyping success

We estimated the AICc values for the 63 possible models trying to explain probability of successfully genotyping individuals of different species using DNA from eggshell swabbing material (dependent variable) because of life history traits in the comparative analyses. The best model explaining differences in combined-genotyping success among bird species included a single variable, namely whether the species used nest-lining material. This was the case for both the *initial* and the *final samples*: differences in AICc values between the best model and the next best model was 1.59 and 2.27 for *initial* and *final samples*, respectively. The evidence ratio for the second best model was 2.22 and 3.12 for *initial* and *final samples*, respectively (for model selection see Burnham & Anderson 2002). Interspecific variation in the use of nest-lining material explained a marginally significant (*initial samples*) and a significant proportion (*final samples*) of interspecific variance in genotyping success as shown by the model testing procedure (PGLS, *initial samples* (23 species): adjusted $R^2 = 0.10$, $F_{1,21} = 3.33$, $P = 0.08$; *final samples* (22 species) adjusted $R^2 = 0.25$, $F_{1,20} = 8.18$, $P = 0.010$). In both models, the degree of phylogenetic dependence (λ) was significantly smaller than one, indicating that differences in genotyping success were not explained by phylogenetic relationships among the species (*initial samples*: $\chi^2 = 5.51$, $P = 0.019$; *final samples*: $\chi^2 = 3.92$, $P = 0.048$).

Discussion

We were able to collect suitable DNA from the target species to be amplified through a multiplex PCR for all 23 species birds tested in this study. However, the amount of DNA obtained was in most cases very low. The success of this technique differed among species, which is at least partially explained by the presence/absence of lining material in the nest. In addition, we found that the effectiveness of eggshell swabbing did not vary depending on the time elapsed after egg was laid. Nonetheless, we also found evidence of genotyping errors (allelic dropout or false alleles) and contamination from another individual than the mother in several of the species tested, regardless of time of egg incubation. Below, we discuss these results and the possibility of the effective use of the eggshell swabbing method for collecting DNA in a wide range of bird species.

Schmaltz *et al.* (2006) proposed and tested this method for recently laid eggs because they may carry a higher quality and a larger quantity of maternal DNA and also because this reduced the probability of contamination with male DNA. Our results, however, suggest that DNA of sufficient quality can be collected throughout incubation. This is possibly because of the continuous adhesion of skin cells from the incubating individuals. In agreement with this possibility, we found DNA present from more than one individual in our egg swab samples (indicated by a) loci displaying 3 or more alleles, b) heterozygous individuals for the *Z-054* locus and c) genotypes resulting from both parents when comparing nestling and egg swab samples in magpies).

In addition to the possible contamination of DNA from other individuals, we have also checked for the presence of possible genotyping errors (allelic dropout

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and false alleles) in our samples, which are common when the DNA template is at a very low concentration (e.g. noninvasive techniques, Taberlet *et al.* 1996; Taberlet & Luikart 1999). To try to detect these kinds of errors in our samples, we compared genotypes of the same samples from two PCR replicates (PCR-10 and PCR-50) and we compared genotypes from magpie nestlings (DNA isolated from blood cells) and those from swabbing magpie eggshells from the same nest. As a result, we detected these genotyping errors in 44% of comparisons between the two PCR replicates (see Table S2 and Table 3). Moreover, the estimated levels of these genotyping errors in our samples and possible contamination from DNA of males are likely to be underestimated because our markers were monomorphic or had few alleles for some species. Moreover, we could not discern the causes of this error (allelic dropout, false alleles or DNA contamination) because we only performed two PCR replicates. In any event and according to our results, the incidence of these genotyping errors associated with swabbing eggs is predicted to be significant, and thus, they should be closely evaluated in pilot studies using a diverse suite of autosomal or Z-linked makers with a high number of alleles within the study population to minimize the overall level of sharing alleles between individuals (Roon *et al.* 2005). Afterwards, and depending on specific results, researchers should evaluate how to address these genotyping errors. For instance, in those species where DNA contamination is probable (e.g. if the male partner participates in egg incubation) or in those species with cooperative breeding or a high intraspecific brood parasitism rate, researchers could sample only those eggs recently laid and/or use only a single swab to sample each egg, as performed by Schmaltz *et al.* (2006). However, it would reduce the amount of DNA available for analysis and could be problematic in species with small eggs. Multiple PCR replicates per sample should be performed to detect and to solve allelic dropout and false alleles (see Taberlet *et al.* 1996; Waits & Paetkau 2005). We have used a Chelex-based procedure for DNA purification optimized for eggshell swab samples by Martín-Platero *et al.* (2010), which results in diluted samples, and in our case, with low DNA concentrations (most of them below detection limit of our fluorometer). However, the amount and quality of DNA extracted could also be improved by using other current and more expensive procedures (e.g. Tan & Yiap 2009) and/or by including an additional step to remove any possible PCR inhibitors (e.g. Das *et al.* 2009). The amount of target DNA extracted from these samples could be quantified *via* quantitative real time PCR (qPCR). Additionally, anyone using this technique could explore methods in which more DNA can be used in each PCR either by increasing the total volume of the PCR or by increasing the DNA concentration (e.g. possible PCR inhibitors could be removed from samples by using other procedure than the dilution of samples, which was used in this study).

On the other hand, interspecific differences in the rate of successful genotyping were not predicted by species-specific variation in egg surface sampled, clutch sizes or any other variables that could potentially affect to the probability of collection and/or preservation of any cells adhered on egg surface (see Introduction and Methods sections). The unique variable explaining the genotyping success was if a bird species did or did not use lining material in their nests. Nest-lining materials could imply better environmental conditions (i.e. temperature and humidity) for genetic material than nests without lining material (e.g. Hilton *et al.* 2004) and retard DNA degradation on the egg surface. Alternatively, it is known that certain lining materials can inhibit the growth of bacteria (Gwinner & Berger 2005; Peralta-Sanchez *et al.* 2010), and these bacteria could degrade any organic material (including bird DNA) present on the egg surface. The bird species tested in this study included a wide range of breeding habits and specific life traits potentially affecting the likelihood of successfully getting maternal DNA from egg surface. Thus, independent of the detected interspecific variation and because of the high probability of collecting suitable DNA, our results suggest that the eggshell swabbing technique could be successfully used in a large number of bird species.

In summary, our results confirm that the swabbing of eggshells is a noninvasive methodology applicable across to a wide range of bird species, including those species where the presence of blood smears is not common and including those species with small eggs. This methodology appears to perform better in those bird species using lining material in the nest, and it can be performed at any time while the eggs are in the nest. In spite of the genotyping errors found, which should be specifically addressed before conducting any extensive sampling, we believe that our results can be important for multiple applications in other bird species. Furthermore, when the distinction between maternal or paternal parent is not needed, this technique provides a useful noninvasive technique in which to collect parental DNA for birds.

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References

Avise JC (2004) *Molecular Markers, Natural History and Evolution*, 2nd edn. Sinauer Associates, Sunderland, Mass.

Bello N, Francino O, Sanchez A (2001) Isolation of genomic DNA from feathers. *Journal of Veterinary Diagnostic Investigation*, **13**, 162–164.

Brown MB, Brown CR (2009) Blood sampling reduces annual survival in cliff swallows (*Petrochelidon pyrrhonota*). *The Auk*, **126**, 853–861.

Burnham KP, Anderson DR (2002) *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*, 2nd edn. Springer, New York.

Bush KL, Vinsky MD, Aldridge CL, Paszkowski CA (2005) A comparison of sample types varying in invasiveness for use in DNA sex determination in an endangered population of greater sage grouse (*Centrocercus uropihasianus*). *Conservation Genetics*, **6**, 867–870.

Criscuolo F (2001) Does blood sampling during incubation induce nest desertion in the female common eider *Somateria mollissima*? *Marine Ornithology*, **29**, 47–50.

Das A, Spackman E, Pantin-Jackwood MJ, Suarez DL (2009) Removal of real-time reverse transcription polymerase chain reaction (RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of avian influenza virus by RT-PCR. *Journal of Veterinary Diagnostic Investigation*, **21**, 771–778.

Dawson DA (2007) Genomic analysis of passerine birds using conserved microsatellite loci. PhD Thesis. University of Sheffield, UK.

Dawson DA, Horsburgh GJ, Küpper C *et al.* (2010) New methods to identify conserved microsatellite loci and develop primer sets of high cross-species utility – as demonstrated for birds. *Molecular Ecology Resources*, **10**, 475–494.

Derrickson KC, Warkentin IG (1991) The role of egg-capping in the evolution of eggshell removal. *The Condor*, **93**, 757–759.

Egloff C, Labrosse A, Hebert C, Crump D (2009) A nondestructive method for obtaining maternal DNA from avian eggshells and its application to embryonic viability determination in herring gulls (*Larus argentatus*). *Molecular Ecology Resources*, **9**, 19–27.

Freckleton RP, Harvey PH, Pagel M (2002) Phylogenetic analysis and comparative data: a test and review of evidence. *The American Naturalist*, **160**, 712–726.

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*). *Journal of Field Ornithology*, **80**, 183–192.

Griffith SC, Owens IPF, Thuman KA (2002) Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology*, **11**, 2195–2212.

Guerrini M, Barbanera F (2009) Noninvasive genotyping of the red-legged partridge (*Alectoris rufa*, Phasianidae): semi-nested PCR of mitochondrial DNA from feces. *Biochemical Genetics*, **47**, 873–883.

Gwinner H, Berger S (2005) European starlings: nestling condition, parasites and green nest material during the breeding season. *Journal of Ornithology*, **146**, 365–371.

Hackett SJ, Kimball RT, Reddy S *et al.* (2008) A phylogenomic study of birds reveals their evolutionary history. *Science*, **320**, 763–1768.

Handel CM, Pajot LM, Talbot SL, Sage GK (2006) Use of buccal swabs for sampling DNA from nestling and adult birds. *Wildlife Society Bulletin*, **34**, 1094–1100.

Hansell MH (2000) *Bird Nests and Construction Behaviour*. Cambridge University Press, Cambridge.

Harvey MG, Bonter DN, Stenzler LM, Lovette IJ (2006) A comparison of plucked feathers versus blood samples as DNA sources for molecular sexing. *Journal of Field Ornithology*, **77**, 136–140.

Hilton GM, Hansell MH, Ruxton GD, Reid JM, Monaghan P (2004) Using artificial nests to test importance of nesting material and nest shelter for incubation energetics. *The Auk*, **121**, 777–787.

Holleley CE, Geerts PG (2009) Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *BioTechniques*, **46**, 511–517.

Idaghdour Y, Broderick D, Korrida A (2003) Faeces as a source of DNA for molecular studies in a threatened population of great bustards. *Conservation Genetics*, **4**, 789–792.

MacDougall D, Amore FJ, Cox GV *et al.* (1980) Guidelines for data acquisition and data quality evaluation in environmental chemistry. *Analytical Chemistry*, **52**, 2242–2249.

Marrero P, Fregel R, Cabrera VM, Nogales M (2009) Extraction of high-quality host DNA from feces and regurgitated seeds: a useful tool for vertebrate ecological studies. *Biological Research*, **42**, 147–151.

Martínez JG, Soler JJ, Soler M, Møller AP, Burke T (1999) Comparative population structure and gene flow of a brood parasite, the great spotted cuckoo (*Clamator glandarius*), and its primary host, the magpie (*Pica pica*). *Evolution*, **53**, 269–278.

Martín-Gálvez D, Soler JJ, Martínez JG *et al.* (2006) A quantitative trait locus for recognition of foreign eggs in the host of a brood parasite. *Journal of Evolutionary Biology*, **19**, 543–550.

Martín-Gálvez D, Dawson DA, Horsburgh GJ, Burke T (2009) Isolation, characterization and chromosome locations of polymorphic black-billed magpie *Pica pica* (Corvidae, AVES) microsatellite loci. *Molecular Ecology Resources*, **9**, 1506–1512.

Martín-Platero AM, Peralta-Sánchez JM, Soler JJ, Martínez-Bueno M (2010) Chelex-based DNA isolation procedure for the identification of microbial communities of eggshell surfaces. *Analytical Biochemistry*, **397**, 253–255.

Martins EP, Hansen TF (1997) Phylogenies and the comparative method: a general approach to incorporating phylogenetic information into the analysis of interspecific data. *The American Naturalist*, **149**, 646–667.

Maurer G, Beck N, Double MC (2010) A 'feather-trap' for collecting DNA samples from birds. *Molecular Ecology Resources*, **10**, 129–134.

Miño CI, Del Lama SN (2009) Molted feathers as a source of DNA for genetic studies in waterbird populations. *Waterbirds*, **32**, 322–329.

Mueller C, Jenni-Eiermann S, Blondel J *et al.* (2006) Effect of human presence and handling on circulating corticosterone levels in breeding blue tits (*Parus caeruleus*). *General and Comparative Endocrinology*, **148**, 163–171.

Nicholls JA, Double MC, Rowell DM, Magrath RD (2000) The evolution of cooperative and pair breeding in thornbills *Acanthiza* (Pardalotidae). *Journal of Avian Biology*, **31**, 165–176.

Nota Y, Takenaka O (1999) DNA extraction from urine and sex identification of birds. *Molecular Ecology*, **8**, 1237–1238.

Pagel M (1999) Inferring the historical patterns of biological evolution. *Nature*, **401**, 877–884.

Paradis E, Claude J, Strimmer K (2004) APE: analyses of phylogenetics and evolution in R language. *Bioinformatics*, **20**, 289–290.

Pearce JM, Fields RL, Scribner KT (1997) Nest materials as a source of genetic data for avian ecological studies. *Journal of Field Ornithology*, **68**, 471–481.

Pemberton JM, Slate J, Bancroft DR, Barrett JA (1995) Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology*, **4**, 249–252.

Peralta-Sanchez JM, Møller AP, Martín-Platero AM, Soler JJ (2010) Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: an experimental study. *Functional Ecology*, **24**, 426–433.

Piggott MP, Taylor AC (2003) Remote collection of animal DNA and its applications in conservation management and understanding the

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population biology of rare and cryptic species. *Wildlife Research*, **30**, 1–13.

R Development Core Team (2009) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Regnaut S, Lucas FS, Fumagalli L (2006) DNA degradation in avian faecal samples and feasibility of non-invasive genetic studies of threatened capercaillie populations. *Conservation Genetics*, **7**, 449–453.

Richardson DS, Jury FL, Dawson DA, Salgueiro P, Komdeur J, Burke T (2000) Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in Sylviidae species and their cross-species amplification in other passerine birds. *Molecular Ecology*, **9**, 2155–2234.

Robertson BC, Minot EO, Lambert DM (1999) Molecular sexing of individual kakapo, *Strigops habroptilu*s Aves, from faeces. *Molecular Ecology*, **8**, 1349–1350.

Roon DA, Thomas ME, Kendall KC, Waits LP (2005) Evaluating mixed samples as a source of error in non-invasive genetic studies using microsatellites. *Molecular Ecology*, **14**, 195–201.

Saladin V, Bonfils D, Binz T, Richner H (2003) Isolation and characterization of 16 microsatellite loci in the great tit *Parus major*. *Molecular Ecology Notes*, **3**, 520–522.

Schmaltz G, Somers CM, Sharma P, Quinn JS (2006) Non-destructive sampling of maternal DNA from the external shell of bird eggs. *Conservation Genetics*, **7**, 543–549.

Segelbacher G (2002) Noninvasive genetic analysis in birds: testing reliability of feather samples. *Molecular Ecology Notes*, **2**, 367–369.

Sheldon LD, Chin EH, Gill SA, Schmaltz G, Newman AEM, Soma KK (2008) Effects of blood collection on wild birds: an update. *Journal of Avian Biology*, **39**, 369–378.

Sibley CG, Ahlquist JE (1990) *The Phylogeny and Classification of Birds: A Study in Molecular Evolution*. Yale University Press, New Haven.

Snow D, Perrins CM, Cramp S (1998) *The Complete Birds of the Western Palearctic on CD-ROM*. Oxford University Press, Oxford.

Sugiura N (1978) Further analysis of data by Akaike's information criterion and finite corrections. *Communications in Statisics - Theory and Methods*, **7**, 13–26.

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

Taberlet P, Luikart G (1999) Non-invasive genetic sampling and individual identification. *Biological Journal of the Linnean Society*, **68**, 41–55.

Taberlet P, Griffin S, Goossens B *et al.* (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **24**, 3189–3194.

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. *Trends in Ecology and Evolution*, **14**, 323–327.

Tan SC, Yiap BC (2009) DNA, RNA, and protein extraction: the past and the present. *Journal of Biomedicine and Biotechnology*, **1**, 10. Article ID 574398.

Tinbergen N (1963) The shell menace. *Natural History*, **72**, 28–35.

Trimbos KB, Broekman J, Kentie R, Musters CJM, de Snoo GR (2009) Using eggshell membranes as a DNA source for population genetic research. *Journal of Ornithology*, **150**, 915–920.

Waits LP, Paetkau D (2005) Noninvasive genetic sampling tools for wildlife biologists: A review of applications and recommendations for accurate data collection. *Journal of Wildlife Management*, **69**, 1419–1433.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Combined genotypes from swab samples and made considering all alleles amplified in any of two PCR replicates for each sample and locus.

Table S2 Genotypes from the two PCR replicates on the same swab samples, one diluted ten-fold (PCR-10) and the other fify-fold (PCR-50).

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