Genetic patterns of Black-tailed Godwit populations and their implications for conservation

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CHAPTER 2

Using eggshell membranes as a DNA source for population genetic research

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Abstract

In the context of population genetic research, a faster and less invasive method of DNA sampling would allow genetic diversity and genetic differentiation to be assessed in greater detail and on larger spatial scales. The aim of this study was to investigate the usefulness of eggshell membranes as a DNA source for population genetic research, by addressing eggshell membrane DNA quality, degeneration and cross-contamination. To this end a comparison was made with blood-derived DNA samples. We have demonstrated 100% successful DNA extraction from post-hatched Black-tailed Godwit eggshell membranes as well as from blood samples. Using 11 microsatellite loci, DNA amplification success was 99.1% for eggshell membranes and 97.7% for blood samples. Genetic information within eggshell membrane DNA in comparison to blood DNA was not affected ($F_{ST} = -0.01735$, $P = 0.999$) by degeneration or possible cross-contamination. Furthermore, neither degeneration nor cross-contamination was apparent in total genotypic comparison of eggshell membrane DNA and blood sample DNA. Our research clearly illustrates that eggshell membranes can be used for population genetic research.

Keywords Black-tailed Godwit · Limosa limosa · Population genetics · Microsatellite · eggshell membrane · DNA quality

Introduction

Over the last few decades a growing number of studies have investigated the population dynamics of different (sub-)populations of a species by genetic methods. As a result, knowledge on conservational and evolutionary issues has increased enormously (Coulon *et al.* 2008, Hoglund *et al.* 2009, Manier and Arnold 2005, Milot *et al.* 2008, Tittler *et al.* 2006). The most widely used yardsticks to investigate population dynamics are genetic diversity and genetic differentiation. Patchily structured populations, influenced by gene flow, genetic drift and locally anomalous selection, will result in different patterns of genetic differentiation and genetic diversity. Consequently, these patterns can be used to address issues of population dynamics and potentially ascribe source-sink dynamics, meta-populations and isolation by distance models to the populations under study.

It is often unclear at what spatial scale population dynamics have a prior influence on genetics and although populations might not show any distinct genetic patterns on a local scale, such patterns could still become apparent at larger scales. This is underlined by the informative results on population dynamics obtained in several population genetic studies that sampled DNA on large spatial scales (Coulon *et al.* 2008, Manier and Arnold 2005, Milot *et al.* 2008, Ortego *et al.* 2008).

Most population and conservation genetic studies today use blood samples as a DNA source (Coulon *et al.* 2008, Hoglund *et al.* 2009, Larsson *et al.* 2008, Manier and Arnold 2005, Milot *et al.* 2008, Ortego *et al.* 2008, Otvall *et al.* 2005). Obtaining blood samples is relatively labour-intensive and sampling DNA to investigate genetics at a landscape level could consequently prove difficult. For this reason there is a need for sampling techniques that are easier to implement. Several alternative sampling methods for collecting DNA have been described in the literature, such as hatched eggshell membranes, pellets, egg swabs, droppings and molted feathers (Bowkett *et al.* 2009, Bush *et al.* 2005, Fernando *et al.* 2006, Schmaltz *et al.* 2006, Solberg *et al.* 2006, Taberlet and Fumagalli 1996). While these sampling methods are being used increasingly in genetic studies, they generally result in DNA of poorer quality and quantity (Taberlet 1998). Furthermore, some of these non-invasive sampling methods, including those involving eggshells, might be prone to contamination problems with parental DNA, DNA from other nest individuals, and other exogenous DNA (Schmaltz *et al.* 2006, Strausberger and Ashley 2001, Taberlet and Fumagalli 1996, Taberlet 1998).

For the purpose of maternity and sex determination analyses, several studies have successfully isolated DNA from eggshell membranes (Bush *et al.* 2005, Pearce *et al.* 1997, Strausberger and Ashley 2001). These studies used eggshell membranes or swabs of eggshells as a DNA source, thereby examining 1-4 microsatellite loci only (Bush *et al.* 2005, Pearce *et al.* 1997, Strausberger and Ashley 2001). To date, however, eggshell membranes have never been used in population genetic research studies. Moreover, for this purpose the general consensus among scientists is that a greater number of microsatellite loci need to be used (Kalinowski 2002, Kalinowski 2005, Pearce *et al.* 1997).

To our knowledge this is the first time that the usefulness of eggshell membranes for population genetic research has been thoroughly investigated using 11 microsatellite loci. In this study we used DNA extracted from eggshell membranes of Black-tailed Godwits (*Limosa limosa*), employing blood sample DNA from Black-tailed Godwit individuals from the same nests as a control. The Black-tailed

Godwit is a large, relatively long-legged waderbird. Over the last 15 years Black-tailed Godwit numbers have declined by 25% globally, prompting IUCN and Birdlife International to classify the species as Near Threatened in 2006. With 40% of the European population now breeding in the Netherlands, this country plays a pivotal role for this species (Birdlife Int. 2004). In the context of ongoing population genetic research on this Near Threatened species at a landscape level it would be desirable to adopt the least invasive and labour-intensive method of sampling.

To investigate the usefulness of eggshell membranes as a DNA source in population genetic research the following questions needed to be addressed. How often can DNA be successfully extracted from post-hatched Black-tailed Godwit eggshell membranes and how does extraction frequency and quality compare with extraction from DNA from blood? Can eggshell membrane DNA be successfully amplified by PCR using 11 microsatellites, and how does this compare with amplification success in blood samples? Is the genetic information from eggshell membrane DNA samples the same as that from blood DNA samples? Is cross-contamination or DNA degeneration more prominent in eggshell membrane-derived DNA than in blood-derived DNA?

Methods

Study site and genetic sampling

Black-tailed Godwit eggshell membranes and blood samples from hatched chicks were collected from Godwit communities between Makkum and Stavoren in Friesland, the northernmost province of the Netherlands. During incubation, nests were located and visited. To determine the hatching date, the developmental stage of the eggs was determined by the floating method described by Liebezeit *et al.* (2007). Around the hatching date the nests were visited once per day to obtain as many eggshell and blood samples per nest as possible. Blood was stored in 97% alcohol buffer and eggshells in individual plastic bags to minimize post-sampling contamination. Both were stored at minus 70°C for later extraction.

Eggshell membrane and blood sample comparison

A stepwise approach was adopted. First, DNA was extracted from the total number of collected eggshells (47) and blood samples (48) from 18 different nests. Second, through PCR amplification of eggshell membrane samples with different purity values, using 4 microsatellites, the effect of purity on amplification was validated and visualized by loading the samples on a 1.2% agarose gel. Finally, after this validation, from the total number of 47 eggshells and 48 blood samples, 21 eggshell membrane and 20 blood DNA samples from the same 7 nests were selected for microsatellite PCR, based on DNA purity.

The genetic differentiation between the selected 20 blood and 21 eggshell membrane DNA samples was calculated to check whether eggshell membrane DNA harboured the same genetic information as blood samples.

To confirm that complete molecular DNA could be obtained from post-hatched eggshell membranes

we matched genotypes between eggshell membrane and blood sample DNA from chicks from the same nests. In this way we also checked for possible cross-contamination of eggshell membrane DNA with DNA from another chick or adult in the same nest or exogenous DNA. If an eggshell membrane DNA sample had the same genotype over 11 microsatellite loci compared to a given blood sample, this was called a match. Possible matches between blood samples and eggshells were unknown beforehand. However, the chance of a possible match per nest was maximized by using only the nests with as many blood and eggshell membrane samples as possible.

DNA extraction

DNA was extracted from 6-10 µl of blood using the Ammonium Acetate method as described by Richardson *et al.* (2001). The Qiagen Dneasy Tissue Kit (Qiagen 2003) was used to extract DNA from eggshell membranes, with minor modifications as described by Bush *et al.* (2005). Subsequently, we modified this protocol by adding 100 µl Buffer AE instead of 200 µl in step 9 of the Qiagen Animal Tissue Protocol Modification. The incubation step that followed was extended from 5 to 10 minutes. Additionally, after spinning down the column, the supernatant was used a second time on the column to maximize DNA yield. Eggshell membrane-derived DNA samples were used undiluted. DNA quality and quantity were checked twice, using the NanoDrop ND-1000 (Thermo Scientific) for 260/280 ratios and concentration values. Additionally, DNA degeneration in all samples was visualized by running them through a 1.2% agarose gel undiluted and checking for smears. For sizing and quantification approximation of amplification fragments, a GeneRuler 1 kb (Fermentas Life Science) was used.

For optimal PCR amplification, blood samples were diluted to concentrations below 20ng/µl. Eggshell membrane DNA samples were used undiluted straight from the Qiagen DNeasy Tissue Kit extraction, as this already resulted in successful PCR amplification.

Microsatellite genotyping

We used eleven microsatellite loci (LIM3, LIM5, LIM8, LIM10, LIM11, LIM12a, LIM24, LIM25, LIM26, LIM30, LIM33) constructed especially for the Black-tailed Godwit as described by Verkuil *et al.* (submitted). The final volumes of the PCR amplification mix were 11 µl and included 1-5 ng DNA for blood samples or 1-30 ng DNA for eggshell membrane samples, 1.65 mM $MgCl_2$, 2.5 µM dNTPs, 0.5 µM forward primer with M13 extension, 0.5 µM reverse primer, 1 µM fluorescent-labelled M13 primer with the same universal extension as the forward primer, 10x PCR buffer and 0.45 U Taq DNA Qiagen polymerase.

The polymerase chain reaction program used was as described by Verkuil *et al.* (submitted), but with one minor modification: the final PCR step was extended from 5 to 20 min. The resulting PCR products were analyzed using a MegaBace 1000 series (Amersham Biosciences) and allele sizes were assigned using a Fragment Profiler 1.2 (Amersham Biosciences 2003). For each sample, PCR amplification success was noted as the successful percentage of positive genotypes scored over 11 microsatellites. To minimize contamination with exogenous DNA during PCR, pre- and post-PCR pipetting were carried out in different rooms. Additionally, to address these potential contamination problems, tubes without DNA samples were included in every PCR reaction as a negative control.

Statistical analysis

Possible differences in genetic information between the two sample sets were addressed by calculating pairwise F_{ST} estimates over 15,000 permutations and a significance level of 0.05 in Arlequin 3.0 (Excoffier and Schneider 2005). To investigate possible allelic dropout and null allele problems in the eggshell membrane DNA sample set due to possible DNA degeneration, we used Micro-Checker 2.2.3 (van Oosterhout *et al.* 2004) with a 95% confidence interval over 10,000 runs.

To match genotypes between eggshell membrane DNA and blood samples from the same nest, we calculated the minimum and maximum number of successful matches that could be expected in each single nest, assuming no contamination or degeneration problems. These values were obtained from the number of eggshell membranes and blood samples within one nest and were then compared with the original number of eggs present within that nest. For instance, if, 3 eggshells and 4 blood samples were collected from one nest with originally 4 eggs, this resulted in both a minimum and a maximum of 3 expected matches. The observed number of matches per nest was then validated by comparing it with the minimum number of expected matches per nest.

Results

DNA was successfully extracted from all 47 eggshell membranes, with DNA concentrations averaging 248 ng/µl (ranging from 32.7 to 543.68 ng/µl). This demonstrates that all sample concentrations were adequate for PCR purposes. A 260/280 ratio, also termed purity value, of ~1.8 or higher is generally accepted as pure DNA. Our nanodrop spectrophotometer measurements demonstrated pure DNA in 72% of the samples tested according to the 260/280 ratio. DNA purity ranged from 0.44 to 2.11, with a total average of 1.77.

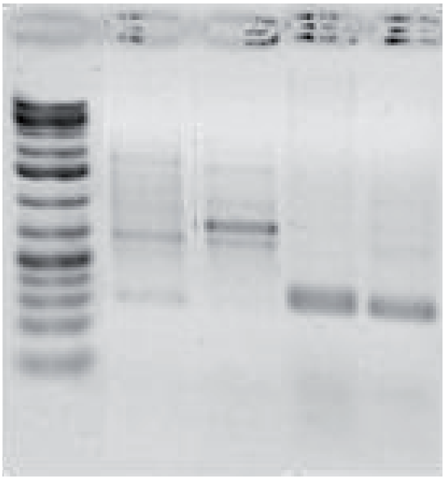

Figure 1. Amplification bands of 4 eggshell membrane DNA samples with different purity values using 1 microsatellite locus. The first lane shows the GeneRuler 1 kb, the 2nd and 3rd lanes samples with respective purity values of 1.5 and 1.35, and the 4th and 5th lanes samples with respective purity values of 2.06 and 2.1.

As can be seen in Figure 1, DNA samples with purity values of 1.5 or lower showed aspecific amplification with several different bands within a lane, while samples with purity values over 2.0 showed single and clear amplification bands. A purity value of 1.6 or higher was regarded as DNA of high purity, which we used for our microsatellite PCR. Some (5) eggshell membrane samples with a DNA purity below 1.6 were extracted a second time. After this second extraction 3 out of 5 eggshell membrane samples gave a DNA purity of >1.6. As a consequence, the percentage of samples with pure DNA rose to 79% and the mean DNA purity value to 1.83. Blood sample DNA extractions were also successful for all 48 samples. The DNA concentrations found averaged 317 ng/µl (with a range of 33.77 to 1051.36 ng/µl). Our nanodrop spectrophotometer measurements demonstrated DNA of high purity in 100% of the samples tested according to the 260/280 ratio. DNA purity ranged from 1.86 to 1.99, with a total average of 1.94. We selected 21 eggshell membrane and 20 blood DNA samples of high purity (Table 1) from the same 7 nests.

Table 1. Eggshell membrane and blood DNA samples with their concentrations and purity, PCR amplification success (PCRamp), Minimum expected matches (Exp min Matches), Maximum expected matches (Exp Max Matches) and observed matches per nest.

Eggshell membrane sample	Purity	PCRamp	Blood sample	Purity	PCRamp	Eggs present within the nest	Exp min Matches	Exp Max Matches	Observed Matches
1.1	2.06	100%	1.1	1.94	82%	4	1	2	1
1.2	2.02	100%	1.2	1.97	100%				
1.3	2.03	100%							
2.1	2.11	100%	2.1	1.92	100%	5	0	2	0
2.2	1.97	100%	2.2	1.95	82%				
			2.3	1.94	100%				
3.1	2.01	100%	3.1	1.95	100%	4	3	3	3
3.2	2.06	100%	3.2	1.94	100%				
3.3	2.09	100%	3.3	1.93	100%				
3.4	2.06	100%							
4.1	2.08	100%	4.1	1.90	100%	4	2	2	2
4.2	2.1	100%	4.2	1.94	100%				
			4.3	1.97	91%				
			4.4	1.95	100%				
5.1	1.76	100%	5.1	1.95	100%	4	3	3	3
5.2	1.67	100%	5.2	1.93	100%				
5.3	1.84	82%	5.3	1.94	100%				
5.4	1.93	100%							
6.1	1.96	100%	6.1	1.98	100%	4	2	3	2
6.2	1.95	100%	6.2	1.96	100%				
6.3	2.09	100%	6.3	1.89	100%				
7.1	1.94	100%	7.1	1.97	100%	4	1	2	2
7.2	1.99	100%	7.2	1.95	100%				
7.3	2.11	100%							

One eggshell membrane DNA isolate out of 21 and 3 blood isolates out of 20 did not amplify for 2 and 5 of the 11 microsatellite loci. The remaining 20 eggshell membrane and 17 blood samples amplified for all 11 loci. Theoretically, amplification failure could have occurred in 21 samples x 11 microsatellite loci for the eggshell membrane set and 20 samples x 11 microsatellite loci for the blood sample set. This resulted in successful amplification rates of 99.1% in the total eggshell membrane group and 97.7% in the total blood sample group (Table 1).

Degeneration of eggshell membrane DNA was slight to non-existent and there was no apparent DNA degeneration in the blood samples, as can be seen in Figure 2.

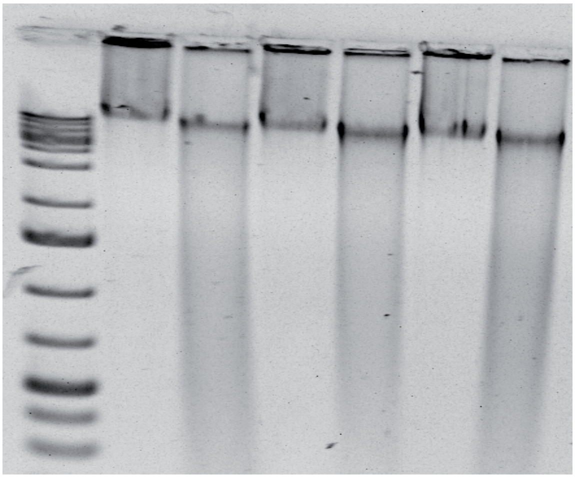

Figure 2. Gel visualizing DNA degeneration of different blood and eggshell membrane samples. The first lane shows the GeneRuler 1 kb, the 2nd lane a blood sample and the 3rd lane an eggshell membrane sample. Subsequent blood and eggshell membrane samples were loaded on the gel in the same order (lanes 4-7).

DNA degeneration could be observed as smears emanating from the bands at the top of the gel, which represent complete molecular DNA. As can be seen in all samples where DNA degeneration occurred, a clear band representing complete molecular DNA could be observed, demonstrating that DNA present in the samples was still of high quality. As expected, blood samples showed no apparent DNA degeneration. This pattern was observed in all the eggshell membrane and blood DNA samples. Using Arlequin 3.0, a F_{ST} value of only -0.01735 was found between the 21 eggshell membrane and 20 blood DNA samples, demonstrating that as a group the eggshell membrane samples were no different (P = 0.999) from the blood samples in terms of the genetic information they contained. Analyses with Micro-checker demonstrated no presence of null alleles or allelic dropout within the eggshell membrane DNA dataset.

The genotypes of the 21 eggshell membrane and 20 blood samples were compared in all 7 nests independently. Table 2 shows the relative PCR amplification success per sample and the minimum expected, maximum expected and observed number of matches. All in all, 12 genotype matches over all 11 microsatellites were observed. In other words, direct linkage of eggshell membrane DNA to a DNA blood sample belonging to a chick was demonstrated in 12 cases. The number of observed genotype matches, over all eleven microsatellites, was never lower than the minimum expected number of matches. This held for all the nests.

Discussion

As eggshells are generally easier to collect than blood samples, using their membranes as a DNA source in population genetic research implemented at the landscape level would represent a clear advantage over the use of blood samples, especially when the subjects under study are relatively rare. As birds do not have to be caught on the nest, intensive visits to the nest are unnecessary to obtain DNA samples. Consequently, more time could be devoted to finding additional nests, which would eventually increase the scale of and genetic detail within the areas sampled. The aim of the present study was to thoroughly evaluate the use of eggshell membranes for this purpose by comparing qualitative and quantitative traits of their DNA to DNA derived from the blood sampling method, which generally results in the highest-quality DNA.

We have demonstrated successful DNA isolation from 100% of the eggshell membranes used. These results compare favourably with those of Bush *et al.* (2005), who achieved a 96% success rate in extraction from eggshell membranes. It may be that the eggshell membranes used in the present study were fresher than those used by Bush *et al.* (2005), leading to fewer extraction failures and higher DNA concentrations. DNA of high purity (>1.6) with good concentrations for PCR was isolated from 79% of the eggshell membranes and 100% of the blood samples. For population genetic research purposes the lower DNA extraction success rate from eggshell membranes might be improved by storing the eggshells at room temperature, which tends to dry out the veined membrane, causing it to separate from the outer shell. This would make it easier to get a good veined membrane sample during the isolation procedure, compared with the use of wet, frozen eggshell membrane samples as indicated by Bush *et al.* (2006) Additionally, the figure of 79% might also be improved by collecting more eggshells from individual nests. In population genetic research it is important to sample as much genetic information as possible. As such, when sampling a population for this kind of research, taking too many samples of individuals that are known in advance to be genetically relatively similar, as with individuals from the same nest, should be avoided. In this view it would be enough to have only one successfully isolated DNA sample per nest to capture most of the genetic information within a population, provided that an adequate number of nests per population are sampled. As such, collecting more eggshells from a nest would increase the chance of DNA isolation from one individual per nest resulting in DNA of high purity. With this method of sampling, 100% of all 17 nests used in this study resulted in at least one DNA isolate generated from eggshell membranes with a DNA purity value of >1.6. In this way the 79% success rate of DNA extraction from eggshell membranes suitable for microsatellite PCR should not pose any problems at all for population genetic research.

We demonstrated PCR amplification success in 99.1% of the eggshell membrane samples, with only 1 eggshell membrane isolate out of 21 not amplifying for two microsatellite loci. As amplification success was lower in the total blood sample set (97.7%), this amplification failure of respectively 0.9 % and 2.3% was seen as a chance PCR event. This shows that eggshell membrane DNA isolates did not yield DNA with more amplification problems than blood DNA isolates. Additionally, failed amplification was not correlated with the purity values of these samples (Table 2). In all likelihood, rerunning specific microsatellite loci for the failed samples within both the eggshell membrane and the blood sample group would generate even higher total successful amplification rates.

When comparing DNA sample sets from different populations of varying DNA quality for the purpose of population genetic research, DNA degeneration and cross-contamination may cause genetic diversity and thus genetic structure values (F_{ST}) to be affected. As became clear from Figure 2, DNA from eggshell membranes exhibited a certain amount of DNA degeneration. As such, we wished to establish whether the DNA from 21 eggshell membranes harboured the same genetic information over 11 microsatellite loci as that from 20 blood samples from the same 7 nests. The genetic differentiation between the two groups was extremely minor and highly non-significant, showing that the two DNA sets were genetically the same. This indicates that even if the eggshell membrane group was affected by degeneration or cross-contamination, this had no influence on genetic differentiation (F_{ST}). Additionally, as most studies use 10 or more microsatellite loci to calculate genetic differentiation and diversity values (Coulon *et al.* 2008, Larsson *et al.* 2008, Ortego *et al.* 2008, Manier and Arnold 2005), these results make clear that eggshell membrane DNA can be effectively used to calculate these values for the purpose of population genetic research.

However, while genetic diversity and F_{ST} values were clearly unaffected by degeneration and possible cross-contamination prominence, we were keen to assess these issues more precisely within eggshell membrane DNA. This was done by comparing the genotypes from eggshell membrane and blood samples taken from individuals from the same 7 nests over 11 microsatellite loci. Within these nests a total of 12 matching genotypes over 11 microsatellite loci were found. The observed number of matches was never lower than the minimum expected number of matches per nest. Additionally, no allelic dropout or null allele problems were observed. This illustrates that DNA degeneration and cross-contamination were not a serious issue.

Summarizing, eggshell membranes taken from eggshells are a very good alternative to blood samples for the purpose of population genetic research. In this context the minor differences in DNA quality between eggshell membrane DNA and blood DNA are insignificant. Our results show, for the first time, that eggshell membrane DNA can be very useful as a DNA source for this type of research.

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