Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/22110 holds various files of this Leiden University dissertation

Author: Trimbos, Krijn
Title: Genetic patterns of Black-tailed Godwit populations and their implications for conservation
Issue Date: 2013-11-05

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

by Krijn Trimbos

Trimbos, Krijn

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

PhD thesis Leiden University, The Netherlands

Cover design and Lay-out : René Glas (www.reneglas.com)
Photo's : Krijn Trimbos
Printed by : Wöhrmann Print Service, Zutphen

ISBN: 978-94-6203-452-5

2013, ALL RIGHTS RESERVED.

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

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 5 november 2013
klokke 15:00 uur
door

Krijn Baptist Trimbos

geboren te Leiden
in 1979

promotie commissie

Promotores	• Prof. dr. Geert R. de Snoo (Dean of the Faculty of Science, CML, University of Leiden) • Prof. dr. Theunis Piersma (Universiteit Groningen, NIOZ)
Co-promotor	• Dr. Kees J.M. Musters (CML, University of Leiden)
Overige leden	• Prof. dr. Nico M. van Straalen (VU Amsterdam) • Prof. dr. Jacob Höglund (Uppsala University) • Prof. dr. Frank Berendse (Wageningen University)

contents

Chapter	title	page
1	General introduction	7
2	Using eggshell membranes as a DNA source for population genetic research	23
3	Intronic variation on the CHD1-Z gene in Black-tailed Godwits *Limosa limosa limosa*: correlations with fitness components revisited	37
4	No evident spatial genetic structuring in the rapidly declining Dutch Black-tailed Godwit *Limosa l. limosa* population	53
5	Genetic differentiation between Dutch Black-tailed Godwit breeding sites re-examined using D statistics	69
6	Patterns in nuclear and mitochondrial DNA reveal historical and recent isolation in the Black-tailed Godwit (*Limosa limosa*)	77
7	General discussion	103
	Summary	119
	Samenvatting	123
	Curriculum Vitae	129
	Publications	133

CHAPTER

1

General Introduction

Introduction

The 20th century intensification of agricultural land use in Europe has lead to steep increases in agricultural yield (de Snoo *et al.* 2012, Stoate *et al.* 2001; 2009). In The Netherlands, most of the low-productive grassland areas have been converted to highly productive agricultural grasslands by applying artificial fertilizer and pesticides, by lowering groundwater levels, and by sowing nutritionally demanding fast growing varieties of grass (de Snoo *et al.* 2012, Stoate *et al.* 2001; 2009). Consequently, flora and fauna diversity in many grassland ecosystems have decreased dramatically (Beintema *et al.* 1995, Bijlsma *et al.* 2001, Donald *et al.* 2001;2006). In this thesis the Black-tailed Godwit *Limosa limosa limosa*, a flagship Dutch farmland bird, was chosen as a model organism to further investigate 2nd and 3rd order effects on biodiversity as a result of the agricultural industrial revolution in the 20th century.

The Black-tailed Godwit is a rather large wader, which can be distinguished by their long bills and legs, the white lower rump contrasting with a black tail-tip and the white stripes across the dark upper wings (http://en.wikipedia.org/wiki/Black-tailed_Godwit - cite_note-collins-2). The Black-tailed Godwit breeds from late March to late June. Three subspecies have currently been described, *L. l. islandica*, *L. l. limosa*, and *L. l. melanuroides*. The nominate *L. l. limosa* breeds from Great Brittain and Central Europe to Central Asia and Asiatic Russia as far east as the river Yenisey (Cramp & Simmons 1982). The islandic subspecies breeds in Iceland, small populations occur on the Faeroe Islands, Shetland and the Lofoten Islands. The *L. l. melanuroides* breeds in Mongolia, Northern China and the Russian Far East.

After the *L. l. limosa* young fledge, breeding birds begin to congregate in flocks, which migrate southwards between late-June and October. During the autumn migration Black-tailed Godwits may roost in large flocks (sometimes tens of thousands) in rice fields in Spain and Portugal, and a proportion of the adults pause in North Morocco in July to moult (Cramp & Simmons 1982). The Black-tailed Godwit winter across a broad range from the Republic of Ireland to Australia, encompassing the Mediterranean, sub-Saharan Africa, and parts of the Middle East, India, China, Taiwan, the Philippines, Indonesia, and Papua New Guinea (Birdlife International 2012). West European Black-tailed Godwits of the *limosa* subspecies winter in Africa North of the equator and south of the Sahel, Guinea-Bissau, the coastal zone of Southern Senegal, the Senegal delta, Mali at the Inner Niger Delta, the Chad Basin (Zwarts *et al.* 2009). An increasing number of West European Black-tailed Godwits winter in Spain and Portugal (Lourenço *et al.* 2010). East European breeding Black-tailed Godwits winter in the eastern part of the Sahel and East Africa (Cramp & Simmons 1982, Zwarts *et al.* 2009). Northward migration takes place between February and April, and birds often arrive at the breeding grounds in large groups.

Current status with a Dutch historical perspective

The European stronghold of the *L. l. limosa* subspecies is found in The Netherlands where 40,000-50,000 Black-tailed Godwit breeding pairs were estimated to breed in 2004 (Birdlife International 2004). In 2004 breeding numbers of *L. l. limosa* were estimated at at 162,000-183,000 in western Europe, 90,000-165,000 in eastern Europe, 25,000-100,000 in West-Central Asia, 150,000 in

Central Asia and Russia (Birdlife International 2004). A still increasing population of 47,000 pairs of L. l. *islandica* breeds in Faeroes, Lofoten and Iceland (Gunnarsson *et al.* 2005). The *L. l. melanuroides* populations are estimated at 160,000 breeding pairs that reside in parts of Eastern Asia (Birdlife International 2012). Until the Middle Ages Black-tailed Godwits probably were not very abundant in The Netherlands. Archeological studies did find bones, in small amounts, of Black-tailed Godwit in food material from 700 B.C. until 1700 (Prummel & Heinrich 2005, Zeiler & Brinkhuizen 2003). Before the Middle Ages Black-tailed Godwit breeding habitat in the Netherlands consisted mainly of raised bogs, moorlands, lake margins, damp grassy depressions in steppe (Beintema *et al.* 1995, Haverschmidt 1963). When raised bogs were converted to wet grasslands and hay lands, during and after the Middle Ages, for the purpose of dairy farming, the Black-tailed Godwit probably shifted from their remaining original breeding habitat to these newly formed habitats (Beintema *et al.* 1995, Haverschmidt 1963).

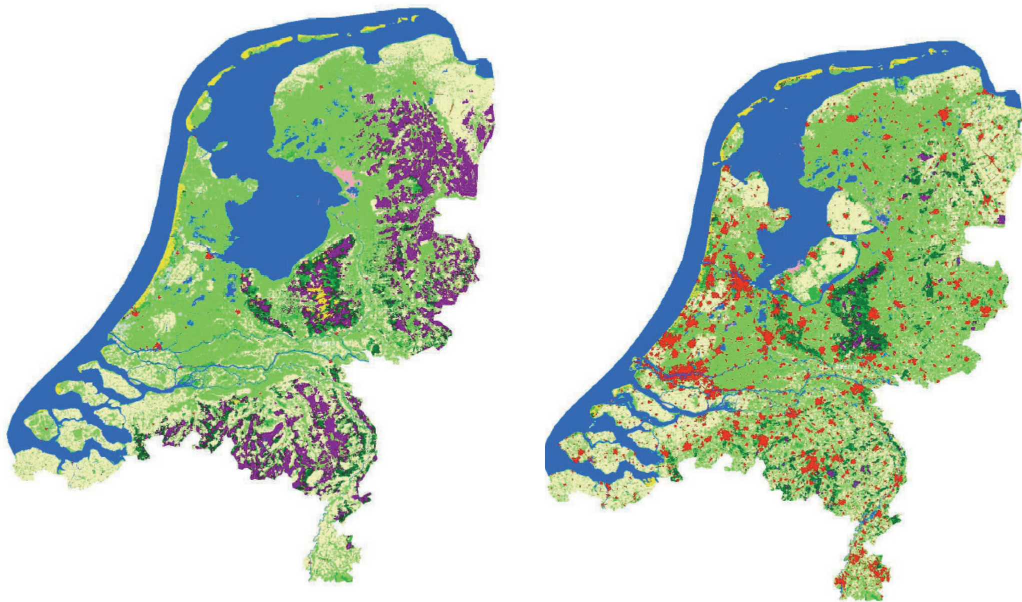


Figure 1: Land use in The Netherlands during 1900 on the left, and 2000 on the right (Knol *et al.* 2004). Colours depicted indicate: grass/hayland (light green), arable/unused land (sand colour), heath/raised bogs (purple), forest (dark green), city/roads (red), dunes (yellow), swamp (pink), water (blue), remaining (grey).

From that time onwards Black-tailed Godwits would be known as breeding birds of meadows: meadow birds. One of the first Dutch ecologists, J. Drijver wrote in 'De Levende Natuur' in 1914: "I highly appreciate the Black-tailed Godwit as a meadowbird…". From 1900 on the Black-tailed Godwit was mostly present as a breeding bird of the damp hay lands, which were the major part of The Netherlands (figure 1). Here the Black-tailed Godwit profited from the usage of artificial fertilizer, in turn increasing food availability, which increased between 1900 and the 1980s (CBS 2001;2008,). This was probably the reason for the increase in Black-tailed Godwit population numbers in these habitats during the first half of the 20th century (Beintema *et al.* 1995, Haverschmidt 1963, Schroeder *et al.* 2010). However, with further agricultural changes population numbers stagnated in the late 1960s when the first comprehensive survey of this breeding bird in The Netherlands yielded an estimate of 120,000 pairs of Black-tailed Godwits in The Netherlands (Mulder 1972). Population numbers have declined ever since with an annual rate estimated at 5% (Bijlsma *et al.* 2001, Gill *et al.* 2007). The available information about Black-tailed Godwit trends indicates that the global population has probably declined between14% and 33% over the past 15 years (Birdlife International 2012). Consequently, the IUCN has currently qualified the species as Near Threatened (Birdlife International 2012).

Reasons for the decrease

Agricultural intensification at the breeding areas is deemed by far the most important negative influence on Black-tailed Godwit breeding numbers (Gill *et al.* 2007, Beintema *et al.* 1995, Hustings & Vergeer 2002, Schroeder *et al.* 2010, Kentie *et al.* 2011). However, several other factors outside and at the breeding areas, such as hunting, predation, urbanization and the decrease of agricultural grassland area due to the conversion of grassland to cornfields, have probably also impacted Black-tailed Godwit population numbers negatively during the second half of the 20th century .

1) Outside the breeding area: hunting

During migration and wintering, hunting poses an additional threat to Black-tailed Godwit adults and fledglings (van Noordwijk & Thomson 2008, Zwarts *et al.* 2009). In France it used to be allowed to shoot Black-tailed Godwit individuals during migration, however since 2008 the EU has implemented a five year ban on hunting the species (Birdlife International 2012). Zwarts *et al.* (2009) report that there is a general increase in the number of Balanta rice farmers in Guinea-Bissau that perceive Black-tailed Godwits as a pest. Since many Black-tailed Godwits now return to Africa earlier, that damages the local crops especially rice crops. This has led to some birds being shot in their wintering grounds (Zwarts *et al.* 2009). However, the precise scale and impact of Black-tailed Godwit hunting in West-Africa are still unknown. Additionally, drought in the West African wintering quarters may have had negative impacts on the mainland European population (Zwarts *et al.* 2009). As a result of drought, foraging opportunities decrease which makes it easier for hunters to shoot Black-tailed Godwits on their West African wintering grounds (Zwarts *et al.* 2009).

2) At the breeding area: decrease of habitat quality

In regards to meadowbirds, agricultural intensification can be summed up by the following: the lowering of the ground water tables, increased sowing of fast growing grass species, higher fertilizer usage, the usage of more life stock, earlier mowing dates and the increase of mowing frequencies

(Beintema *et al.* 1995, Bijlsma *et al.* 2001, Birdlife International 2004, Donald *et al.* 2001;2006). Agricultural intensification practices are all directed towards increasing productivity. Lower groundwater tables negatively affect the availability of prey items for Black-tailed Godwit adults and chicks during the breeding season (Kleijn *et al.* 2009;2010, Verhulst *et al.* 2007).

Earthworms which form the most important food source for adult Black-tailed Godwits generally follow the ground water table (Siepel *et al.* 1990). When ground water tables are lowered earthworms become less available for the foraging birds (Kleijn *et al.* 2009). This might cause adults to postpone breeding, which in turn might negatively influence egg size during nesting (Schroeder *et al.* 2012). Moreover, several studies show that small egg size is negatively correlated with chick body condition and survival (Arnold *et al.* 2006, Blomqvist *et al.* 1997, Bolton 1991). Dry soil warms up quicker, resulting in a faster growth of the grass vegetation. In combination with using fast growing grass species, high fertilizer input, and the introduction of the monoculture, the grass swards forms tall and dense swards which negatively affects foraging capabilities for Black-tailed Godwit chicks (Kleijn *et al.* 2010, Schekkerman *et al.* 2008). Production benefits from this denser and higher grass vegetation as it allows for earlier and more frequent mowing during spring. Agricultural mechanization has further increased production allowing for faster mowing of larger meadows. As a result current mowing dates have advanced, while the timing of breeding has stayed the same.This poses a direct danger for meadowbird reproduction, see figure 2 (Beintema *et al.* 1995, Kleijn *et al.* 2010, Musters *et al.* 2011).

Furthermore, mowing has been demonstrated to decrease the availability of arthropod abundance in the freshly mown fields, increasing chick mortality through starvation (Kleijn *et al.* 2010, Schekkerman & Beintema 2007). An additional problem with earlier mowing is the reduced cover that chicks have to deal with on the freshly mown fields. This might increase chick predation significantly (Schekkerman *et al.* 2009, Teunissen *et al.* 2005). Moreover, the increase of meadowbird predators, Northern Goshawk (*Accipiter gentilis)*, Common Buzzard (*Buteo buteo*), small carnivorous mammals and probably the Red Fox (*Vulpes vulpes*) in general might also negatively influence Black-tailed Godwit recruitment (Teunissen *et al.* 2005; 2008). Like in large parts of the world, urbanization in The Netherlands has increased dramatically over the last 100 years (figure 1). Increased urbanization might further increase predation as potential predators like jackdaws (*Corvus monedula*), house cats and dogs have easier excess to the meadows and as such decrease habitat quality.

3) At the breeding area: habitat fragmentation

As a result of decreasing habitat quality the areas with breeding Black-tailed Godwit have become fragmented. Moults and skins of the Black-tailed Godwit collected between 1826-1935 show that surprisingly many individuals were found in or close to the present day core breeding areas (Schroeder *et al.* 2009; figure 3a). Black-tailed Godwit breeding areas seem to be mostly connected in 1967 (figure 3b). Currently, it is clear that the Black-tailed Godwit breeding landscape is better described by fragments of breeding sites surrounded by unoccupied areas (figure 3c;d). Small fragments of habitat can only support small populations of plants and animals. Furthermore, small populations are more vulnerable to extinction. Minor fluctuations in climate, resources, or other factors could be catastrophic in small, isolated populations where they would have been insignificant and quickly

corrected for in large populations (Hanski 1999). For mobile species, like birds, the dangers of habitat fragmentation are generally seen as less important. However, the effect that habitat fragmentation can have on the population dynamics of a species is not only dependent on the mobility but also on the breeding site faithfulness of a species (Hanski 1999). The Black-tailed Godwit is a highly breeding site faithful bird with high adult nest site fidelity and some natal philopatry (Groen 1993, Kentie *et al.* 2011, Kruk *et al.* 1998, van den Brink *et al.* 2008). Groen (1993) showed 90% of the adult breeding birds returning within 700 m of the previous nest site. Natal philopatry was demonstrated as well with 75% of the birds returning with 18 km of their previous hatching site. Kruk *et al.* (1998) showed that on the basis of ring recovery data from the Dutch ringing center between 1900 and 1991 nearly 3% of the natal philopatry took place beyond 50 km. This combination of high breeding site faithfulness and continuing habitat fragmentation of Black-tailed Godwit breeding populations could pose a serious threat to the long term survival of the species.

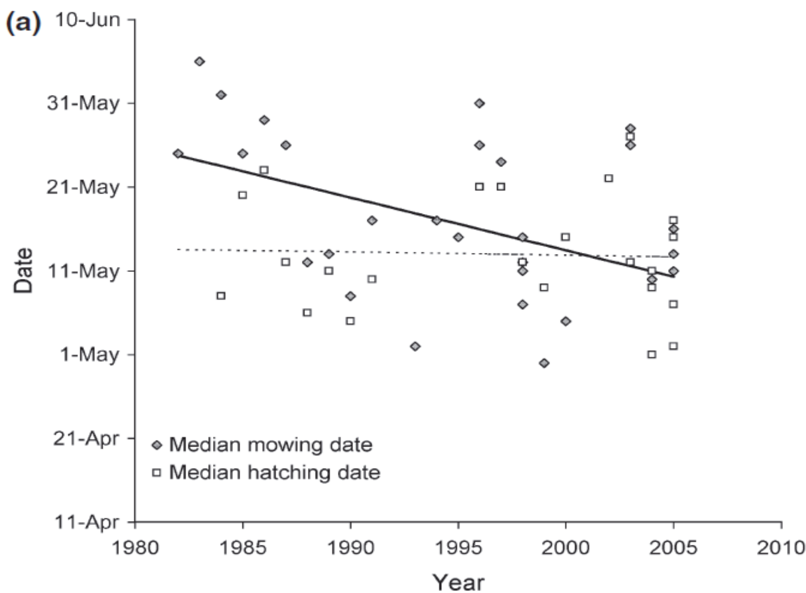


Figure 2. The median hatching date of the Black-tailed Godwit and the median mowing date of dairy farm grasslands in The Netherlands from 1980 to 2005 (Kleijn *et al.* 2010).

Urbanization, next to the increased planting of trees and vegetation encroachment in the agricultural meadows which might decrease habitat quality, might further influence Black-tailed Godwit populations negatively as it decreases landscape openness and the agricultural grassland areal (Melman *et al.* 2008, van der Vliet *et al.* 2008;2010). This might further increase habitat fragmentation for breeding Black-tailed Godwits. It remains unclear if this combination of high breeding site faithfulness and the increasing amount of habitat fragmentation is currently causing isolation of Black-tailed Godwit breeding areas.

Figure 3: Black-tailed Godwit distribution through time a) The distribution of Black-tailed Godwits between 1826-1935, destillated from moults and skins of Black-tailed Godwits that were collected on different locations in the breeding season during this time, b) the Black-tailed Godwit distribution map from 1967 (Mulder 1972), c) the Black-tailed Godwit distribution map from 2005 (based on the ´Gruttokaart´ Teunissen *et al.* 2005), d) a local Black-tailed Godwit distribution map from the southwestern part of Fryslan 2011 (Kentie *et al.* 2011).

4) The proportion of the population affected by the decrease

It seems that the current negative Black-tailed Godwit population trends mainly results from the decreased recruitment during breeding. Research on Black-tailed Godwit adult survival in The Netherlands demonstrates an absence of clear changes over the last 25 years (Roodbergen *et al.* 2008;2012, Schekkerman *et al.* 2008). Demographic studies conducted on Dutch Black-tailed Godwit populations, which estimated adult survival and which kept sample size and ringing effort stable throughout the study period, demonstrated adult survival in recent years to be similar to adult survival in 1960, lying around 0.8 (Beintema & Drost 1986, Groen & Hemerik 2002, Kentie *et al.* 2011, Noordwijk & Thomson 2008, Roodbergen *et al.* 2008, Schekkerman *et al.* 2008). Contrastingly, like Black-tailed Godwit population trends, chick recruitment has been showing a steady decrease since 1980 (Schekkerman *et al.* 2008).

Altogether, these agricultural changes, urbanization and decrease of available grassland areal, seem to have led to the decrease in habitat quality and the fragmentation of suitable grassland, in turn decreasing Black-tailed Godwit recruitment (Gill *et al.* 2007, Kentie *et al.* 2013, Schekkerman *et al.* 2008, Zwarts *et al.* 2009).

Policies to stop the decrease

To halt and reverse negative Black-tailed Godwit population trends, several management strategies have been formulated and have been implemented throughout the 20th century. In 1909 the conservation agency 'Natuurmonumenten' already instigated grassland reserves for the purpose of meadow bird preservation (Beintema *et al.* 1995). Since then the surface of grassland reserves have increased enormously (Dekker 2002). A second form of meadow bird management is the protection of the nests by volunteers. Nest protection increases clutch success by twofold and is especially effective for nests found on grassland and crop farms that would otherwise be destroyed by agricultural activities and trampled by cattle (Oosterveld *et al.* 2007). Presently, over 150,000 meadowbird nests are protected by volunteers (Goedhart *et al.* 2010). Around 2000, an alternative management technique was introduced to stop the rapid decline of meadowbirds in the Netherlands, called 'mosaic management', as it became increasingly clear that increasing clutch survival alone could not halt the negative Godwit population trends. Higher recruitment was needed, which could be obtained by providing suitable foraging habitat for adults during the nesting period and chicks after hatching; grassland with high water tables for foraging adults and grass with the suitable density and height supplying chicks with enough food and cover, up to fledging (Oosterveld *et al.* 2011). The most important characteristics of 'mosaic management' are an implementation on a landscape level and a specific spatial distribution of this grassland mosaic within the area, with the purpose to meet all the ecological requirements of the meadow birds throughout the breeding season (Oosterveld *et al.* 2011). Several forms of mosaic management, such as postponed mowing, adapted grazing, an increase of the amount of water on the production land to increase foraging possibilities for meadowbirds and using hay rich farmyard manure instead of semi liquid manure to increase a more open and lower meadowbird grassland habitat have been used in different compositions throughout The Netherlands. Farmers that agree to join mosaic management are compensated for the loss in production (Oosterveld *et al.* 2011).

Lack of knowledge

It remains questionable if these different management strategies are adequate to sustain Black-tailed Godwit populations. As a management type, buying and managing new grassland reserves optimally for meadow birds has slowed down. Nest protection is an adequate measure to increase clutch success in many cases but clutch success alone does not improve recruitment. Furthermore, in areas with high predation pressure nest protection often may have negative effects on clutch survival (Goedhart *et al.* 2010). It was shown that the number of nest visits increases predation success by nearly 10% per visit in areas with high predation pressure since voluntary nest protectors leave trails or body scents directed towards the nests, which might be easy to follow by predators (Goedhart *et al.* 2010). Furthermore, mowing around protected nests leaves little islands of grass around these nests in an otherwise mown field, which might also increase predation success (Kentie *et al.* 2011). Mosaic management results (which in The Netherlands form an important part of the agri-environmental schemes) are still difficult to interpret, as its efficiency is highly dependent on the combination of mosaic management types implemented, the focal species, the weather conditions during the breeding season and local external factors like predation pressure and the amount of disturbance (Kleijn & Sutherland 2003, Kleijn *et al.,* 2006, Oosterveld *et al.* 2007;2011, Verhulst *et al.,* 2007, Willems *et al.,* 2004). Up till now it has still not been scientifically demonstrated that these measurements have the desired effect (Oosterveld *et al.* 2011). However, to know whether mosaic or any other form of conservation management has effect on meadowbird populations on the landscape level, one needs to determine if these measurements can improve habitat quality in terms of recruitment and consequently decrease habitat fragmentation.

By using capture mark recapture techniques (CMR) a broader understanding of the quantity and consequence of increasing habitat fragmentation and decreasing habitat quality can be acquired. CMR techniques can be used to study population dynamic aspects like, source-sink dynamics, dispersal, breeding site faithfulness, natal philopatry, the fraction of birds that breed and yearly survival of the population, which in turn can be used to quantify habitat fragmentation or determine habitat quality (Bouwhuis *et al.* 2012, Kentie *et al.* 2011, McGowan *et al.* 2009). CMR studies come at the cost of being time consuming, which limits the spatial and temporal scale across which they can be carried out. This may hinder the extrapolation to non studied areas.

Another way to study the effects of habitat fragmentation and habitat quality is through genetics. Habitat fragmentation and changes in habitat quality might affect the population dynamics of a species. In turn the resulting population dynamic processes, such as a metapopulation structure or sources and sinks might have an effect on the genetic pattern of the species. Accordingly, these genetic patterns can be studied through conservation genetics. Conservation genetics focuses on the genetic consequences arising from reduction of once large, outbreeding populations to small units, as a result of for instance deteriorating habitat quality or habitat fragmentation, where stochastic factors and the effects of inbreeding are profound. It encompasses the use of genetic theory, derived from the field of population genetics. Through the field of conservation genetics, genetic processes such as inbreeding, genetic drift, the loss of genetic diversity, and gene flow can be studied, all of

which might be informative about the population dynamics of the species under study (Frankham *et al.* 2010). Subsequently, this type of genetic research can help with the reasoning of why and how a species should be protected. This can be done through resolving taxonomic uncertainties, defining management units, resolving fragmented population structures, identifying species or populations at risk due to low genetic diversity and defining sites for reintroduction (Frankham *et al.* 2010). As it is much less labour-intensive than the marking and following of cohorts of individual animals over their lifetime, conservation genetic research can be conducted on larger spatial scales. This might lead to a better understanding of habitat fragmentation and quality on the landscape level. Additionally, genetic changes that can potentially result from habitat fragmentation and changes in habitat quality take place during a much longer time frame than those that can be researched with CMR studies. This could increase the insight in historical population changes when using genetics.

The aim of this thesis was to use genetics to investigate long term population dynamic processes in Black-tailed Godwit populations resulting from increasing habitat fragmentation or isolation of different breeding habitat on three different spatial scales. However, before genetic research could be conducted genetic markers and easy sampling methods were needed. Therefore, microsatellite markers were developed. Additionally, the potential of a DNA source other than blood was investigated for the rest of the genetic research. After this methodological research this thesis focuses on answering the next questions.

1. *Can eggshell membranes be used as a reliable DNA source in genetic research?*
2. *What are the genetic differences between breeding populations on intensively managed agricultural grassland and extensively managed agricultural grassland?*
3. *What are the genetic differences between geographically isolated breeding populations in The Netherlands?*
4. *What are the genetic differences between breeding populations on a global scale?*

To investigate this we focus on three spatial scales: regional, national and global.

Outline of the thesis

This thesis is composed of 6 research chapters and a general discussion.
In **chapter 2** we used the developed microsatellite markers to test egg shell membranes as an alternative sampling method to blood samples in population genetic research. Collecting egg shell remains is less time consuming than collecting blood samples and more DNA samples can be gathered, by collecting egg shells, during the same time span compared to blood samples. We focused on contamination issues, the DNA yield and purity of egg shell membrane derived DNA and compared the results to DNA isolated from blood samples.

With the developed markers and additional sampling method generated from these methodological studies, further genetic research could be conducted. These will be discussed in chapters 4 to 6. In **chapter 3** we re-examine the very interesting findings of a previous study that demonstrated intronic variation in the CHD1-Z gene in Black-tailed Godwits to be correlated with fitness using a much bigger dataset based on additional years of the previous CMR study. The previous study suggested that Black-tailed Godwit breeding populations were differentially structured at different habitat types as a result of positive selection pressures on birds breeding at extensively managed (generally considered to be of high habitat quality to Black-tailed Godwits) agricultural grassland. Here we re-examined these findings of possible population structure correlated with habitat quality on a local scale. Furthermore, we investigated several other fitness correlates.

Chapter 4 describes the results of the population genetic structure of Dutch Black-tailed Godwit breeding populations. Nine different breeding locations entailing most of the Black-tailed Godwit breeding range in The Netherlands were analyzed for population structure, F-statistics, genetic diversity, genetic differentiation, inbreeding, isolation and gene flow.

Chapter 5 re-examines the genetic differentiation between these nine Black-tailed Godwit breeding sites using a relatively new form of statistics called D. This statistic is less dependent on heterozygosity and within population diversity to determine genetic differentiation between populations and as such might yield different results compared to the traditional F-statistics.

In **chapter 6**, the genetic population structure and phylogeny of the Black-tailed Godwit was assessed, entailing DNA samples ranging from Iceland to Lake Baikal in eastern Russia and including all three subspecies of Black-tailed Godwit. A historic explanation is given for the genetic patterns found in both microsatellites and newly developed mtDNA markers.

Finally, in **chapter 7** the results and subjects of the previous chapters are discussed. In this general discussion we explore the usefulness of our findings and population genetic structure studies for the conservation of Black-tailed Godwit populations and animal conservation in general.

References

Arnold JM, Hatch JJ, Nisbet ICT (2006) Effects of egg size, parental quality and hatch-date on growth and survival of Common Tern *Sterna hirundo* chicks. Ibis, 148, 98-105.

Blomqvist D, Johansson OC, Gotmark F (1997) Parental quality and egg size affect chick survival in a precocial bird, the Lapwing *Vanellus vanellus*. Oecologia, 110, 19-24.

Bolton M (1991) Determinants of chick survival in the lesser black-backed gull: relative contributions of egg size and parental quality. Journal of Animal Ecology, 60: 949-960.

BirdLife International (2012) Species factsheet: *Limosa limosa*. Downloaded from http://www.birdlife.org on 06/01/2012.

Birdlife International (2004) Birds in Europe: population estimates, trends and conservation status. Cambridge, UK.

Beintema AJ, Drost N (1986) Migration of the Black-tailed Godwit. Gerfaut, 76, 37-62.

Beintema A, Moedt O, Ellinger D (1995) Ecologische Atlas van de Nederlandse Weidevogels. Schuyt & Co, Haarlem

Bijlsma, RG, Hustlings, F, Camphuysen, CJ (2001) Algemene en Schaarse Vogels van Nederland (Avifauna van Nederland 2). GMB Uitgeverij/KNNV Uitgeverij, Haarlem/Utrecht.

Bouwhuis S, Choquet R, Sheldon BC, Verhulst S (2010) The forms and fitness cost of senescence: age-specific recapture, survival, reproduction, and reproductive value in a wild bird population. The American Naturalist, 197, E15-E27.

CBS (2001) Tweehonderd jaar statistiek in tijdreeksen 1800-1999. Centraal Bureau voor de Statistiek, Voorburg/Heerlen.

CBS (2008) Land/ en tuinbouwcijfers 2008. Centraal Bureau voor de Statistiek, Voorburg/Heerlen.

Cramp S, Simmons KEL (1982) The Birds of the Western Palearctic. Vol III. Oxford: Oxford University press.

Donald PF, Green RE, Heath MF (2001) Agricultural intensification and the collapse of Europe's farmland bird populations. Proceedings of the Royal Society B, 268, 25-29.

Donald PF, Sanderson FJ, Burfield IJ, van Bommel FPJ (2006) Further evidence of continent-wide impacts of agricultural intensification on European farmland birds, 1990-2000. Agriculture Ecosystems & Environment, 116, 189-196.

Drijver J (1914) De Grutto (*Limosa limosa*). De Levende Natuur, 19, 5-9.

Frankham R, Ballou JD, Briscoe DA (2010) Introduction to Conservation Genetics. Cambridge, UK.

Gill JA, Langston RHW, Alves JA, Atkinson PW, Bocher P, Cidraes Vieira N, Chrockford NJ, Gelinaud G, Groen N, Gunnarsson TG, Hayhow B, Hooijmaijer J, Kentie R, Kleijn D, Lourenco P, Masero, JA, Meunier F, Potts P, Roodbergen M, Schekkerman H, Schroeder J, Wymenga E, Piersma T (2007) Contrasting trends in two black-tailed godwit populations: A review of causes and recommendations. Wader Study Group Bulletin, 114, 43-50.

Goedhart PW, Teunissen WA, Schekkerman H (2010) Effect van nestbezoek en onderzoek op weidevogels. Ministerie van LNV, Directe Kennis, Den Haag.

Groen NM, Hemerik L (2002) Reproductive success and survival of Black-tailed Godwits *Limosa limosa* in a declining local population in The Netherlands. Ardea, 90, 239-248.

Groen NM (1993) Breeding site tenacity and natal philopatry in the Black-tailed Godwit. Ardea, 81, 107-113.

Haverschmidt F (1963) The black-tailed godwit. E.J. Brill. Leiden

Hanski I (1999) Metapopulation ecology. Oxford University Press

Hustings F, Vergeer JW (2002) Atlas van de Nederlandse Broedvogels 1998-2000 verspreding aantallen verandering. National Natuurhistorisch Museum Naturalis, KNNV Uitgeverij.

Kentie R, Hooijmeijer JCEW, Both C, Piersma T (2011) Weidevogels in ruimte en tijd 2007-2010. Eindrapport. Ministerie van LNV, Directe Kennis, Den Haag.

Kentie R, Hooijmeijer JCEW, Trimbos KB, Groen NM, Piersma T (2013) How dairy farming practices affect growth and survival of meadow bird chicks. Journal of Applied Ecology, 50, 243-251.

Kleijn D, Sutherland WJ (2003) How effective are European agri-environment schemes in conserving and promoting biodiversity? Journal of Applied Ecology, 40, 947-969.

Kleijn D, Baquero RA, Clough Y, Diaz M, De Esteban J, Fernández F, Gabriel D, Herzog F, Holzschuh A, Jöhl R, Knop E, Kruess A, Parshall EJP, Steffan-Dewenter I, Tscharntke T, Verhulst J, West TM, Yela JL (2006) Mixed Biodiversity benefits of agri-environment schemes in five European countries. Ecology Letters, 9, 243-254.

Kleijn D, Dimmers W, van Kats R, Melman D (2009). Het belang van hoog waterpeil en bemesting voor de grutto: I. De Vestigingsfase, Ii. De Kuikenfase. De Levende Natuur 110: 180-184.

Kleijn D, Schekkerman H, Dimmers WJ, Kats RJM, Melman D, Teunissen WA (2010) Adverse effects of agricultural intensification and climate change on breeding habitat quality of black-tailed godwits *Limosa l. limosa* in the Netherlands. Ibis, 152, 475-486.

Knol WC, Kramer H, Gijsbertse H (2004) Historisch grondgebruik Nederland: een landelijke reconstructie van het grondgebruik rond 1900. Alterra, Wageningen, Alterra-rapport 573.

Kruk M, Noordervliet MAW, Ter Keurs WJ (1998) Natal philopatry in the black-tailed godwit *Limosa limosa* L. and its possible implications for conservation. Ringing and Migration, 19, 13–16

Lourenço PM, Kenti R, Schroeder J, Alves JA, Groen NM, Hooijmeijer JCEW, Piersma T (2010) Phenology, stop-over dynamics and population size of migrating black-tailed godwits *Limosa limosa* at a key staging area, the Tejo and Sado rice plantations. Ardea, 98, 35-42.

McGowan CP, Millspaugh JJ, Ryan MR, Kruse CD, Pavelka G (2009) Estimating survival of precocial chicks during the prefledging period using a catch-curve analysis and count-based age class data. Journal of Field Ornithology, 80, 79-87.

Melman T, Schotman A, Hunink S, de Snoo G (2008) Evaluation of meadow bird management, especially black-tailed godwit (*Limosa l. limosa*), in The Netherlands. Journal of Nature Conservation, 16, 88-95.

Mulder T (1972) De grutto (*Limosa limosa* (L.)) in Nederland: aantallen, verspreiding, terreinkeuze, trek en overwintering. Bureau van de K.N.N.V., Hoogwoud.

Musters CJM, Snoo GR de, Keurs WJ ter (2010) Timing of the breeding season of Black-tailed Godwit *Limosa Limosa* and Northern Lapwing *Vanellus vanellus* in the Netherlands. Ardea, 98, 195-202.

Oosterveld EB, Terwan P, Guldemond JA (2007) Mozaïekbeheer voor weidevogels: evaluatie en mogelijkheden voor optimalisering. Ministerie van LNV, Directie Kennis, Den Haag.

Oosterveld, EB, Nijland F, Musters CJM, de Snoo GR (2011) Effectiveness of spatial mosaic management for grassland breeding shorebirds. Journal of Ornithology, 152, 161-170.

Prummel, W. & D. Heinrich, 2005. Archaeological evidence of former occurrence and changes in fishes, amphibians, birds, mammals and molluscs in the Wadden Sea area. Helgoland Marine Research, 59, 55-70.

Roodbergen M, Klok TC, Schekkerman H (2008) The ongoing decline of the breeding population of Black-Tailed Godwits *Limosa l. limosa* in The Netherlands is not explained by changes in adult survival. Ardea, 96, 207-218.

Roodbergen M, van der Werf B, Hotker H (2012) Revealing the contributions of reproduction and survival to the Europe-wide decline in meadow birds: review and meta-analysis. Journal of Ornithology, 153, 53-74.

Rosenzweig, ML(1995) Species diversity in space and time. Cambridge: Cambridge University Press.

Schekkerman H, Beintema AJ (2007) Abundance of invertebrates and foraging success of Black-tailed Godwit *Limosa limosa* chicks in relation to agricultural grassland management. Ardea, 95, 39-54.

Schekkerman H, Teunissen WA, Oosterveld E (2008) The effect of 'mosaic management' on the demography of Black-tailed Godwit *Limosa limosa* on farmland. Journal of Applied Ecology, 45, 1067-1075.

Schmitt T (2007) Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. Frontiers in Zoology, 4, 1-13.

Schroeder J, Lourenço PM, Hooijmeijer JCEW, Both C, Piersma T (2009) A possible case of contemporary selection leading to a decrease in sexual plumage dimporphism in a grassland-breeding shorebird. Behavioral Ecology, 20, 797-807.

Schroeder J, Piersma T, Groen NM, Hooijmeijer JCEW, Kentie R, Lourenço PM, Schekkerman H, Both C (2012) Reproductive timing and investment in relation to spring warming and advancing agricultural schedules. Journal of Ornithology, 153, 327-336.

Snoo GR de, Naus N, Verhulst J, van Ruijven J, Schaffers AP (2012) Long-term changes in plant diversity of grasslands under agricultural and conservation management. Applied Vegetation Science, 15, 299-306.

Stoate C, Baldi A, Beja P, Boatman ND, Herzon I, van Doorn A, de Snoo GR, Rakosy L, Ramwell C (2009) Ecological impacts of early 21st century agricultural change in Europe – A review. Journal of Environmental Management, 91, 22-46.

Stoate C, Boatman ND, Borralho RJ, Rio Carvalho C, de Snoo GR, Eden P (2001) Ecological impacts of arable intensification in Europe. Journal of Environmental Management, 63, 337-365.

Teunissen W, Altenburg W, Sierdsema H (2005) Toelichting op de gruttokaart van nederland 2004. SOVON Vogelonderzoek Nederland, Beek-Ubbergen. Altenburg & Wymenga ecologisch onderzoek bv. Veenwouden, SOVON-onderzoeksrapport 2005/04, A&W-rapport 668.

Teunissen W, Schekkerman H, Willems F (2005) Predatie bij weidevogels. Op zoek naar mogelijke effecten van predatie op de weidevogelstrand. SOVON Vogelonderzoek Nederland, Beek-Ubbergen. Alterra, Wageningen, SOVON-onderzoeksrapport 2005/11. Alterra-Document 1292.

Teunissen W, Schekkerman H, Willems F, Majoor F (2008) Identifying predators of eggs and chicks of Lapwing *Vanellus vanellus* and Black-tailed Godwit *Limosa limosa* in The Netherlands and the importance of predation on wader reproductive output. Ibis, 150, 74-85.

van den Brink V, Schroeder J, Both C, Lourenco PM, Hooijmeijer JCEW, Piersma T (2008) Space use by Black-tailed Godwits *Limosa limosa limosa* during settlement at a previous or a new nest location. Bird Study, 55, 188-193.

van der Vliet RE, Schuller E, Wassen MJ (2008) Avian predators in a meadow landscape: consequences of their occurrence for breeding open-area birds. Journal of Avian Biology, 39, 523-529.

van der Vliet RE, van Dijk J, Wassen MJ (2010) How different landscape elements limit the breeding habitat of meadow bird species. Ardea, 98, 203-209.

van Noordwijk AJ, Thomson DL (2008) Survival rates of Black-tailed Godwits *Limosa limosa* breeding in The Netherlands estimated from ring recoveries. Ardea, 96, 47-57.

Verhulst J, Kleijn D, Berendse F (2007) Direct and indirect effects of the most widely implemented Dutch Agri-environmental schemes on breeding waders. Journal of Applied Ecology, 44, 70-80.

Willems F, Breeuwer A, Foppen R, Teunissen W, Schekkerman H, Goedhart P, Kleijn, D, Berendse F (2004) Evaluatie Agrarisch Natuurbeheer: effecten op weidevogeldichtheden. Rapport 2004/02 SOVON Vogelonderzoek Nederland, Wageningen Universiteit en Researchcentrum, Wageningen, The Netherlands.

Zeiler JT, Brinkhuizen DC (2003) Resten van rijke maaltijden: archeozoologisch onderzoek van botmateriaal uit de Krijtstaat te Gorinchem (14e – 17e eeuw). ArchaeoBone rapport nr. 35. ArchaeoBone, Leeuwarden, The Netherlands.

Zwarts L, Bijlsma RG, van der Kamp J, Wymenga E (2009) Living on the edge; wetlands and birds in a changing Sahel. KNNV Publishing, Zeist.

CHAPTER

2

Using eggshell membranes as a DNA source for population genetic research

Journal of Ornithology (2009) 150, 915-920

Krijn B. Trimbos, Joyce Broekman,
Rosemarie Kentie, C.J.M. Musters, Geert R. de Snoo

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.

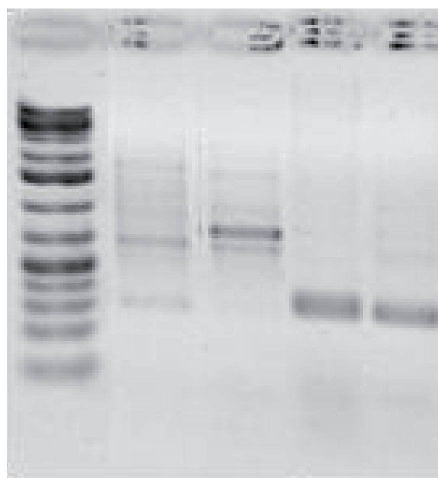


Figure 1. Amplification bands of 4 eggshell membrane DNA samples with different purity values using 1 microsatellite locus. The first lane shows the GeneRuler 1 kb, the 2nd and 3rd lanes samples with respective purity values of 1.5 and 1.35, and the 4th and 5th lanes samples with respective purity values 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%				
1.2	2.02	100%	1.2	1.97	100%	4	1	2	1
1.3	2.03	100%							
2.1	2.11	100%	2.1	1.92	100%				
2.2	1.97	100%	2.2	1.95	82%	5	0	2	0
			2.3	1.94	100%				
3.1	2.01	100%	3.1	1.95	100%				
3.2	2.06	100%	3.2	1.94	100%	4	3	3	3
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	2.1	100%	4.2	1.94	100%	4	2	2	2
			4.3	1.97	91%				
			4.4	1.95	100%				
5.1	1.76	100%	5.1	1.95	100%				
5.2	1.67	100%	5.2	1.93	100%	4	3	3	3
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%				
6.2	1.95	100%	6.2	1.96	100%	4	2	3	2
6.3	2.09	100%	6.3	1.89	100%				
7.1	1.94	100%	7.1	1.97	100%				
7.2	1.99	100%	7.2	1.95	100%	4	1	2	2
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.

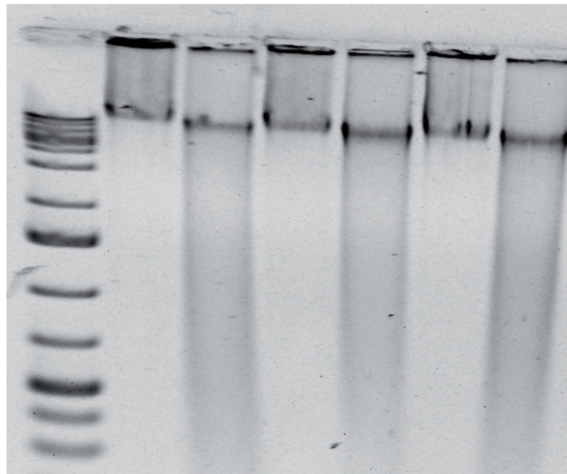


Figure 2. Gel visualizing DNA degeneration of different blood and eggshell membrane samples. The first lane shows the GeneRuler 1 kb, the 2nd lane a blood sample and the 3rd lane an eggshell membrane sample. Subsequent blood and eggshell membrane samples were loaded on the gel in the same order (lanes 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 membranes can be very useful as a DNA source for this type of research.

Acknowledgments

We would like to thank Theunis Piersma, Jos Hooijmeijer, Petra de Goeij, Pedro Lourenco and the rest of the Groningen University Black-tailed Godwit group for their help in collecting eggshells and blood samples, storing the eggshells and providing laboratory space for DNA extractions. Marco van der Velde from the University of Groningen was of great help with these extractions. Yvonne Verkuil from the Groningen University helped with understanding the Arlequin and Micro-checker software. Klaas Vrieling and Rene Glas of the Leiden Institute of Biology (IBL) were helpful with PCR amplifications and providing laboratory space. We are also grateful to Nigel Harle for brushing up our English.

References

Birdlife International (2004) Birds in Europe: population estimates, trends and conservation status. Cambridge, UK.

Bowkett, AE, Plowman AB, Stevens, JR, Davenport TRB, van Vuuren BJ (2009) Genetic testing of dung identification for antelope surveys in the Udzungwa Mountains, Tanzania. Conservation Genetics, 10, 251-255

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 an endangered population of greater Sage-Grouse (*Centrocercus uropihasianus*). Conservation Genetics, 6, 867-870.

Coulon A, Fitzpatrick JW, Bowman R, Stith BM, Makarewich CA, Stenzler LM, Lovette IJ (2008) Congruent population structure inferred from dispersal behaviour and intensive genetic surveys of the threatened Florida scrub-jay (*Aphelocoma coerulescens*). Molecular Ecology, 17, 1685-1701.

Excoffier LGL, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, 1, 47-50.

Fernando P, Polet G, Foead N, Ng LS, Pastorini J, Menick DJ (2006) Genetic diversity, phylogeny and conservation of the Javan rhinoceros (*Rhinoceros sondaicus*). Conservation Genetics, 7, 439-448.

Hoglund J, Johansson T, Beintema A, Schekkerman H (2009) Phylogeography of the Black-tailed Godwit *Limosa limosa*: substructuring revealed by mtDNA control region sequences. Journal of Ornithology, 150, 45-53.

Kalinowski ST (2002) How many alleles per locus should be used to estimate genetic distances? Heredity, 88, 62-65.

Kalinowski ST (2005) Do polymorphic loci require large sample sizes to estimate genetic distances? Heredity, 94, 33-36.

Larsson JK, Jansman HAH, Segelbacher G, Hoglund J, Koelewijn HP (2008) Genetic impoverishment of the last black grouse (*Tetrao tetrix*) population in the Netherlands: detectable only with a reference from the past. Molecular Ecology, 17 ,1897-1904.

Liebezeit JR, Smith PA, Lanctot RB, Schekkerman H, Tulp I, Kendall SJ, Tracy DM, Rodriques RJ, Meltofte H, Robinson JA, Gratto-Trevor C, McCaffery BJ, Morse J, Zack SW (2007) Assessing the development of shorebird eggs using the flotation method: Species-specific and generalized regression models. Condor, 109, 32-47.

Manier MK, Arnold SJ (2005) Population genetic analysis identifies source-sink dynamics for two sympatric garter snake species (*Thamnophis elegans* and *Thamnophis sirtalis*). Molecular Ecology, 14, 3965-3976.

Milot E, Weimerskirch H, Bernatchez L (2008) The seabird paradox: dispersal, genetic structure and population dynamics in a highly mobile, but philopatric albatross species. Molecular Ecology, 17, 1658-1673.

Ortego J, Calabuig G, Aparicio J, Cordero PJ (2008) Genetic consequences of natal dispersal in the colonial lesser kestrel. Molecular Ecology, 17, 2051-2059.

Ottvall R, Hoglund J, Bensch S, Larsson K (2005) Population differentiation in the redshank (*Tringa totanus*) as revealed by mithochondria DNA and amplified fragmenth length polymorphism markers. Conservation Genetics, 6, 321-331.

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.

Qiagen (2003) DNeaasy Tissue Handbook. protocol for isolation of total DNA from animal tussues. pp.18-20, QIAGEN. Vlaencia, California, USA.

Richardson DS, Jury FL, Blaakmeer K, Komdeur J, Burke T (2001) Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). Molecular Ecology, 10, 2263-2273.

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.

Solberg KH, Bellmain E, Drageset OM, Taberlet P, Swenson JE (2006) An evaluation of field and non-invasive genetic methods to estimate brown bear (*Ursus arctos*) population size. Biological conservation, 128, 158-168.

Strausberger BM, Ashley MV (2001) Eggs yield nuclear DNA from egg-laying female cowbirds, their embryos and offspring. Conservation Genetics, 2, 385-390.

Taberlet P, Fumagalli L (1996) Owl pellets as a source of DNA for genetic studies of small mammals. Molecular Ecology, 5, 301-305.

Taberlet P, Waits LP (1998) Non-invasive genetic sampling. Trends in Ecology & Evolution, 13, 26-27.

Tittler R, Fahrig L, Villard MA (2006) Evidence of large-scale source-sink dynamics and long-distance dispersal among wood thrush populations. Ecology, 87, 3029-3036.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes, 4, 535-538.

Verkuil YI, Trimbos K, Haddrath O, Baker AJ Characterization of polymorphic microsatellite DNA markers in the black-tailed godwit (*Limosa limosa: Aves*). Molecular Ecology Resources, 9, 1415-1418.

CHAPTER

3

Intronic variation on the CHD1-Z gene in Black-tailed Godwits *Limosa limosa limosa*: correlations with fitness components revisited

Ibis (accepted)

Krijn B. Trimbos, Rosemarie Kentie, Marco van der Velde, Jos C.E.W. Hooijmeijer, Carola Poley, C.J.M. Musters, Geert R. de Snoo, Theunis Piersma

Abstract

Recently, Schroeder *et al.* suggested that intronic variation in the CHD1-Z gene of Black-tailed Godwits (*Limosa limosa limosa*) breeding in southwest Friesland, The Netherlands, correlated with fitness components. Here we re-examine this surprising result using a much expanded dataset (on 2088 birds from 2004-2010 rather than 284 birds from 2004-2007). We find that the presence of the Z* allele (9% of the birds) does not associate with breeding habitat type, nor with egg size, adult survival, adult body mass or adult body condition. The results presented here, when used in synergy with the previously reported findings, suggest that there might be a tendency towards female adults with the Z* allele laying earlier clutches than adult females without the Z* allele. The occurrence of the Z* allele was associated with a higher chick body mass and return rate. Chicks with the Z* allele that had hatched early in the breeding season were heavier at birth compared to chicks without the Z* allele and chicks with the Z* allele that had hatched late. The present results suggest that neutral variation in the CHD1-Z gene may indeed have arisen as a byproduct of selection in females and during the egg and chick-phases.

Keywords CHD1-Z*, molecular sexing, neutral variation, sample size issues, selection

Introduction

In species where sexes are morphologically difficult to distinguish, molecular intronic markers have been used to assign sex (Griffiths *et al.* 1996; 1998, Ellrich *et al.* 2010, Saino *et al.* 2010). The Chromo-Helicase-DNA Binding (CHD1) gene, present on both the Z (CDH1-Z) and W-chromosome (CDH1-W) in birds, is widely used for this purpose. By simultaneous Polymerase Chain Reaction (PCR), the CDH1-Z and CDH1-W loci are amplified and the length difference between the resulting allele fragments is used to distinguish male and female genotypes. Males will show two allele fragments of the same length (ZZ), whereas females will show allele fragments of different size (ZW). There are an increasing number of studies reporting length variation in the PCR-amplified fragments of the CHD1-Z locus (Dawson *et al.* 2001, Montell *et al.* 2001, Lee *et al.* 2002, Agate *et al.* 2004, Jarvi and Farias 2006, Casey *et al.* 2009, Schroeder *et al.* 2010), variation that is expected to be selectively neutral.

Nevertheless, other studies have reported correlations between intraspecific variation in the CHD1-Z gene and fitness components. In Common Moorhens (*Gallinula chloropus*) a polymorphism in the CHD1-Z gene was associated with increased mortality in heterozygous male chicks (Lee *et al.* 2002). In Ovenbirds a polymorphism in the CHD1-Z gene was found to correlate with male body mass (Toms *et al.* 2012). In Black-tailed Godwits (*Limosa limosa limosa*), normally amplified CHD1-W and CHD1-Z alleles fragment have lengths of 393 and 378 bp, respectively, whereas a polymorphism on the CHD1-Z gene resulted in a third possible allele fragment of 374 bp (Z*) (Schroeder *et al.* 2010). Accordingly, Black-tailed Godwit males have three different genotypes (ZZ, ZZ* or Z*Z*) and females two (ZW, Z*W). Based on a sample of 284 Black-tailed Godwits from Friesland, The Netherlands, the rare Z* allele appeared to be positively associated with several fitness components, but homozygote Z*Z*genotypes were not found. Males and females with the Z* allele had less extensive breeding plumage, and females bred earlier and had larger eggs than birds without the Z* allele. There was also an association with breeding habitat type, such that in areas managed as meadowbird reserves (extensively agricultural land) the Z* allele was found in 14% of the birds whereas no birds with the Z*allele were found in modern, intensively managed agricultural land.

Schroeder *et al.* (2010) proposed three explanations for such intriguing functional correlations with supposedly neutral genetic variation. First, the polymorphism could reflect underlying population structure. Second, the polymorphism might have been directly affecting phenotypic variation. Schroeder *et al.* (2010) indicated that this is not very likely as the CHD1-Z gene has a role in chromatin structure mediation and organization during transcription and should therefore be conserved (Stokes and Perry 1995, Schroeder *et al.* 2010). The third possibility was that this polymorphism is linked to other, non-neutral, loci on the same chromosome.

Here we revisit the story of Schroeder *et al.* (2010) with a much larger sample of 2088 individuals collected in 2004-2010 in the same study area and including the earlier data. Not only was sample size higher, we were also able to look at a greater range of fitness correlates, including chick survival in addition to habitat selection, timing of breeding, egg size and adult survival.

Methods

In late May-June 2004 to 2010 blood samples of individual Black-tailed Godwits were collected in southwest Friesland in a study area that from 2007 onward comprised 8340 hectare of intensively and extensively managed grasslands (see Groen *et al.* 2012 for more details on the study area). Upon finding a nest during incubation, the floating method of Liebezeit *et al.* (2007) was used to predict hatch date, so that around the day of predicted hatch adults and chicks could be captured on the nest. Each adult bird received an individual combination of four colour-rings plus a flag on their tibia for individual recognition. Since 2008 hatchlings (chicks still in the nest) were given an engraved leg-flag: a flag with a written three letter/number combination. Subsequently, different biometric measurements (bill length, total head, wing length, tarsus length, tarsus toe length) and body weight were taken from each individual. In addition to ringing the birds and taking their biometrics, we obtained 30 µl whole blood samples from the brachial vein of adults or the leg vein of chicks. Blood was stored in individual 1.5 ml Eppendorf tubes containing 97% alcohol buffer, after which the individual tubes were frozen at -80 °C.

DNA was extracted from 6-10 µl of blood using the Ammonium Acetate method as described by Richardson *et al.* (2001) or by using Qiagen Dneasy Tissue Kit (Qiagen 2003), with minor modifications as described by Trimbos *et al.* (2009). DNA quality and quantity were checked twice, using the NanoDrop ND-1000 (Thermo Scientific) for 260/280 ratios and concentration values. For optimal PCR amplification, blood samples were diluted to concentrations below 10 ng/µl. We used fluorescently labeled P8 and P2 primers and the PCR amplification protocol as described by Griffiths *et al.* (1998). The PCR products were separated on an ABI 377 automatic sequencer (data from 2004 - 2007) or AB3730 DNA analyzer (data from 2008 - 2010). Subsequently their fragment length was determined using Genescan 3.1 software or Genemapper 4.0 software, respectively.

A total of 2088 individuals, comprising 618 adults and 1470 chicks, were typed for sex and the presence of the Z*allele (Table 1). To be able to compare results between Schroeder *et al.* (2010) and this study, most of the fitness calculations of Schroeder *et al.* (2010) were repeated for more extensive samples that included the ones used by Schroeder *et al*. (2010). We excluded plumage traits from the fitness analyses because of the impossibility to score this in all birds (especially hatchlings).

Table 1. The number of individuals used for Hardy-Weinberg equilibrium and habitat selection calculations are displayed below. The number of Individuals with and without the Z* allele are subdivided by sex, age and habitat type and totals for every subcategory are given.

	Male adult	Male chick	Total	Female adult	Female chick	Total	Intensively managed meadowland	Extensively managed meadowland	Moved between management types	Total
Z	266	658	924	296	683	979	98	363	74	535
Z*	32	92	124	24	37	61	8	34	13	55
Total	298	750	1048	320	720	1040	106	397	87	590

When the Z* allele is indeed positively correlated with different fitness variables, or when the homozygote Z*Z*genotype is lethal, the amount of different genotypes present in the populations might deviate from Hardy-Weinberg expectations (Frankham *et al.* 2009). The sex ratio is expected to be 1:1. However, possible correlates between the CHD1-Z* allele variant and fitness components might cause skew, and we thus verified if sex ratio deviated from expected values. Chi-Square tests were performed to test if the differences between observed and expected values for the number of genotypes, sex ratio, and males and females carrying the Z* allele were significant.

We tested if the occurrence of the rare Z*allele was restricted to individuals breeding on meadows managed for meadow birds (herb-rich meadows', Schroeder *et al.* 2010, Groen *et al*. 2012). Hatchlings and larger chicks were excluded from this analysis. Because some adults were captured in multiple years, we used a generalized linear mixed model (GLMM) with breeding habitat as response variable, individual as random effect, and genotype as fixed effect. In total 708 observations of 590 different adults were included in this analysis (Table 1).

To analyze if the Z*allele had an effect on yearly apparent survival, marked godwits were recaptured and/or resighted with binoculars or spotting telescopes (Lourenço *et al.* 2011). Adults with known genotypes marked in 2004-2009, and resighted from 2005 to 2010 were selected for an analysis of annual apparent survival using a Cormack Jolly Seber (CJS) mark-recapture model in the program MARK (White and Burnham 1999). Of the 461 individuals included in this analysis, 42 carried the Z*allele. An individual was reported alive if it was seen at least twice and on different dates within the breeding season March to June. The full model, was the model in which the survival probability was dependent on year, presence or absence of the Z*allele, and the interaction between year and Z*allele. The resighting probability was able to vary only between years because we think it is unlikely that the Z*allele would influence resighting probabilities. The goodness-of-fit of the global model was tested with the program U-CARE (Choquet *et al.* 2009) and the model fitted the data well (χ^2 =25.7945, df = 26, p = 0.47). Model selection was based on Akaike's Information Criterion (AIC) to select the most parsimonious model (Burnham and Andersson 2002).

Black-tailed Godwits vary greatly in size, so in addition to body mass which is body size dependent, we used a body size independent mass termed 'condition' (van der Meer and Piersma 1994). Of 603 adults body size measurements (bill, total head, wing, tarsus, tarsus toe length), the body mass, molecular sex and CHD1-Z genotype were known. For 'condition' the size parameters were collapsed in a Principal Components Analysis to extract a single size parameter, representing structural body size. The first principal component (PC1) explained 80.8% of the variation. The residuals of the linear regression of body mass and PC1 ($R^2 = 0.70$, $F_{1,601} = 1429$, $p < 0.001$) were taken as a size independent condition measure.

To test for correlations between presence or absence of the CHD1-Z*and body mass and body condition, we used a linear mixed effect model, with sex, the interaction sex and CHD1-Z genotype, habitat quality (modern or herb-rich agricultural land), and date of capture as fixed effects. Because some individuals were captured in multiple years, we used individual as random factor. Additionally, year of capture was used as random effect.

Egg volumes and lay dates (the day that the first egg was layed) of nests with at least one of the parents carrying the Z*allele were compared to egg volumes and lay dates of nests where none of the parents carried the Z*allele. For this analysis we used only nests of which we knew both parents. Hereafter, we analyzed if there was an effect of the occurrence of Z*on egg volume in males and females separately. Similarly, a mixed effect model was used here, with breeding pairs or individuals (pairs are monogamous and no divorces were observed) and year as random effect. For the egg volume analysis we used, lay date, habitat quality and the interaction of lay date with CHD1-Z genotype as fixed effects. The egg volume analysis for two parents per nest resulted in a too small dataset to perform the analysis with year as a random factor, therefore year was included as fixed factor. For the analysis on lay date, habitat quality, the interaction CHD1-Z genotype with habitat quality and the interaction of CHD1-Z genotype with year as fixed effects.

From 2008 to 2010, 914 freshly hatched chicks were marked with an engraved leg flag, enabling us to investigate if there was a difference in return rates of chicks with and without the Z* allele. We used observations of chicks in subsequent years (in either breeding area or on the migration route to increase resightings). Since the quality of the hatching habitat (modern or herb-rich agricultural land) and hatching year might influence chick survival, we included these factors in the analysis. Genotype, year, habitat quality, hatching date, body mass, the interaction genotype and hatching date, the interaction genotype and body mass and the interaction genotype and habitat quality were included in the analyses as fixed effect. We could not assign year as a random factor, as there were only two years included. Because of the low number of resightings and observation years, we used a Generalized Linear Model for binomial data with a logit link, instead of doing a mark-recapture analysis. We also analysed if the CHD1-Z genotype of the chick itself had an influence on its body mass. For this analysis we used the body mass data from 1470 chicks that were caught between 2004 and 2010. To prevent pseudo replication of chicks that were born within the same nest, we analyzed this with a mixed model with nest and year as a random effect. Genotype, sex, habitat quality and hatching date, the interaction genotype and hatching date and the interaction genotype and sex were included in the analyses as fixed effect. We included hatching date as it has been suggested to be correlated with reproductive success (Kruk *et al.* 1997, Arnold *et al.* 2006, Schroeder *et al.* 2012) and might be an important explanatory factor. Differences in sample size between different analyses were caused by missing values of some variables.

Statistics were done in R.2.11.1 (R Development Core Team, 2008) unless stated otherwise. Non-normal variables were transformed. The function lm() was used for the general linear model, lme() for linear mixed effect models, lmer() for the generalized linear mixed effect model and glm() for generalized linear models. For the linear mixed models we performed a manual backward selection using the anova() function for models fitted with the ML method, while the estimates were given after refitting the model with the REML method. The PCA was done with the prcomp() function, using a correlation matrix. Chi square tests were conducted using chisq.test().

Results

Of the 2088 individuals 1048 were male (298 adults and 750 chicks) and 1040 were female (320 adults and 720 chicks), which did not deviate from an expected sex ratio of 1 (X^2 = 0.03, df = 1, *P* = 0.86) (Table 1). 9% of the reported genotypes carried the Z* allele (Table 1). This corresponded to a Z*allele frequency of 6% (q = 0.06, and p = 0.94). We expected 4 males out of 1040 to be Z*Z* given the Z*allele frequency. We encountered one chick with the homozygous Z*Z* genotype; its biometrics were comparable with other chicks (mass 29 gram, bill 17.6 mm). The number of observed genotypes did not differ significantly from the number of genotypes expected under Hardy-Weinberg expectations (Fisher's exact test *P* = 0.78). Eight out of 106 godwits caught on intensive and 34 out of397 godwits caught on herb-rich meadows carried the Z*allele. Additionally, 13 out of 87 godwits that were caught on meadows of both management types throughout the study period, carried the Z* allele (Table 1). There was no difference between the occurrence of the Z* allele in birds in intensively and herb-rich agricultural land (*z* = 0.82, *P* = 0.4).

The model where apparent survival varied per year and resighting probability was constant was the best supported (Table 2). There was no support that the Z* allele had an effect on yearly apparent survival. The most parsimonious model including Z* was the model with an interaction between Z* and year and had a ΔAICc of 7.6. Resighting probability was high (*P* = 0.89, SE=0.01), as was average yearly apparent survival (*phi* = 0.89, SE = 0.004).

Table 2. Model results for the apparent survival of Black-tailed Godwits and its relationship with the Z* allele. Apparent survival (phi) can be year dependent (t), dependent on genotype (Z) or dependent on the interaction Z*t, or not vary over years and genotype (.). Resighting probability (p) can be year dependent (t) or not (.).

Model	AICc	Δ AICc	AICc Weights	Model Likelihood	Num. Par	Deviance
Phi(t) p(.)	**1510.24**	**0.00**	**0.92**	**1.00**	**7**	**168.99**
Phi(t) p(t)	1515.85	5.61	0.06	0.06	11	166.48
Phi(Z*t) p(.)	1517.81	7.58	0.02	0.02	13	164.35
Phi(Z*t) p(t)	1523.44	13.20	0.00	0.00	17	161.76
Phi(.) p(.)	1533.54	23.31	0.00	0.00	2	202.38
Phi(Z) p(.)	1534.85	24.61	0.00	0.00	3	201.68
Phi(.) p(t)	1537.32	27.08	0.00	0.00	7	196.07
Phi(Z) p(t)	1538.63	28.40	0.00	0.00	8	195.36

In the model describing adult body mass (Table 3), only sex remained as a significant term after model selection (t_{595} = -34.97, *P* < 0.001), with males being 56 g lighter than females. Adults with a Z* allele were not heavier than adults without the Z* allele (t_{594} = 0.46, *P* > 0.5). The interaction term sex and genotype was not significant according to the 5% criterion (t_{593} = -1.77, p = 0.08). This interaction compares females without the Z-allele (the intercept) to males with the Z* allele. If the interaction between sex and Z* presence was kept in the model, females with a Z* allele were not

significantly heavier (estimate = 6.02 ± 3.82 SE gram, p = 0.11) than females without a Z* allele, and males with a Z* also did not differ in mass to males without a Z* allele; they were 3.31 ± 4.47 SE gram lighter. Sex was also the only remaining term in the best model for condition (estimate = -7.95 ± 1.49, t_{595} = -5.33, P <0.001), suggesting that females had a higher size-independent mass. Genotype was not maintained in this model (t_{592} = 0.40, P > 0.5). Leaving capture date (t_{594} = -1.80, P = 0.07) in the model did not change the effect of genotype (t_{593} = 1.00, P > 0.5).

Table 3. Model results for the general linear mixed model of CHD1-Z genotype related to body mass and condition of adult male and female Black-tailed Godwits. Terms left in the model are shown in bold/italic, and the terms are shown in reversed order of exclusion from the model. Estimates and statistics were derived from the last model before exclusion. Reference category was: [1] female,[2] Z allele, [3] intensively agriculturally managed.

	Estimate	SE	t value	df	P
Body Mass					
Intercept	***311.07***	***1.28***	***243.47***	***595***	***<0.001***
Sex[1]	***-56.10***	***1.60***	***-34.97***	***595***	***<0.001***
Z[2]	1.21	2.64	0.46	594	>0.5
Z*Sex	-9.33	5.27	-1.77	593	0.08
Habitat quality[3]	2.44	2.12	1.15	591	0.25
Capture date	-0.11	0.09	-1.23	592	0.22
Random effects					
$σ2_{year}$	***1.68***				
$σ2_{individual}$	***18.37***				
$σ2_{residual}$	***6.83***				
Condition					
Intercept	***3.60***	***1.12***	***3.21***	***595***	***0.001***
Sex[1]	***-7.95***	***1.49***	***-5.33***	***595***	***<0.001***
Z[2]	0.98	2.45	0.40	592	>0.5
Capture date	-0.15	0.08	-1.80	594	0.07
Z*Sex	-6.03	4.91	-1.23	591	0.22
Habitat quality[3]	-2.29	1.93	-1.19	593	0.24
Random effects					
$σ2_{year}$	***1.20***				
$σ2_{individual}$	***17.19***				
$σ2_{residual}$	***6.08***				

Table 4. Model results of the general linear mixed model of CHD1-Z genotype related to egg size and lay dates for male and female Black-tailed Godwits (lay date is measured as day since 1st of April). Terms left in the model are shown in bold/italic, and the terms are shown in reversed order of exclusion from the model. Estimates and statistics were derived from the last model before exclusion. Reference categories were: [1] 2004, [2] intensively agriculturally managed, [3] Z allele.

Laydate						Egg size					
	Estimate	SE	t-value	df	P		Estimate	SE	t-value	df	P
Both						**Both**					
Intercept	***5.07***	***0.20***	***25.30***	***115***	***<0.001***	***Intercept***	***40.90***	***0.31***	***131.98***	***97***	***<0.001***
Z[3]	-0.23	0.17	-1.36	114	0.18	Year[1]					
Habitat quality[2]	0.17	0.26	0.65	113	0.5	2005	-1.09	1.05	1.03	20	0.3
Z * habitat quality	-1.26	0.90	-1.40	112	0.16	2006	0.83	0.69	1.19	20	0.2
						2007	-0.71	0.75	-0.95	20	0.4
						2008	0.50	0.82	0.61	20	>0.5
						2009	-1.48	0.94	1.58	20	0.13
						2010	0.71	0.87	0.82	20	0.4
						Z[2]	0.78	0.72	1.08	95	0.3
						Sqrt laydate	-0.01	0.26	-0.04	19	>0.5
						Z * sqrt(laydate)	-0.55	0.57	-0.55	18	>0.5
						Habitat quality[2]	-0.0	0.98	0	94	>0.5
Random effects						**Random effects**					
$\sigma2_{year}$	***0.48***					***$\sigma2_{intercept}$***	***2,65***				
$\sigma2_{individual}$	***0.00***					***$\sigma2_{residuals}$***	***1,61***				
$\sigma2_{residuals}$	***0.84***										
Male						**Male**					
Intercept	***5.43***	***0.15***	***35.53***	***354***	***<0.001***	***Intercept***	***41.13***	***0.42***	***98.97***	***354***	***<0.001***
Habitat quality[2]	***-0.38***	***0.12***	***-3.28***	***8***	***0,01***	Z[3]	0.77	0.43	1.79	353	0.08
Z [3]	-0.06	0.13	-0.48	353	>0.5	Habitat quality[2]	0.41	0.39	1.04	8	0.3
Z * habitat quality	0.28	0.33	0.86	352	0.4	Sqrt laydate	0.02	0.18	0.10	7	>0.5
						Z * sqrt(laydate)	-0.28	0.51	-0.56	6	>0.5
Random effects						**Random effects**					
$\sigma2_{year}$	***0.28***					***$\sigma2_{year}$***	***1.01***				
$\sigma2_{individual}$	***0.47***					***$\sigma2_{individual}$***	***1.40***				
$\sigma2_{residuals}$	***0.67***					***$\sigma2_{residuals}$***	***2.40***				
Female						**Female**					
Intercept	***5.38***	***0.16***	***33.97***	***423***	***<0.001***	***Intercept***	***42.73***	***0.66***	***64.67***	***423***	***<0.001***
Habitat quality [2]	***-0.36***	***0.11***	***-3.23***	***21***	***0.004***	***Sqrt laydate***	***-0.37***	***0.13***	***-2.92***	***21***	***0.01***
Z [3]	-0.28	0.15	-1.93	422	0.05	Habitat quality[2]	-0.06	0.27	-0.22	20	>0.5
Z * habitat quality	-0.86	0.65	-1.32	400	0.19	Z[3]	-0.74	0.53	1.40	422	0.16
						Z * sqrt(laydate)	0.34	0.68	0.50	421	>0.5
Random effects						**Random effects**					
$\sigma2_{year}$	***0.31***					***$\sigma2_{year}$***	***0.14***				
$\sigma2_{individual}$	***0.36***					***$\sigma2_{individual}$***	***2.92***				
$\sigma2_{residuals}$	***0.80***					***$\sigma2_{residuals}$***	***0.89***				

The eggs in nests of which one parent had the Z*allele did not differ in volume compared with eggs in nests where neither of the parents had the Z*allele (t_{95} = 1.08, *P* = 0.3) (Table 4). The occurrence of the CHD1-Z* genotype (Table 4) was not correlated with average egg volume in a separate analysis for male and female (male: t_{353} = 1.79, *P* = 0.08, female: t_{353} = -0.48, *P* > 0.5), while for males eggs differed in volume between years and for females eggs differed between years and were smaller later in the season (Table 4).

Laying dates for nests where only one parent had the Z*allele were not significantly different from nests where neither of the parents carried the Z*allele (t_{114} = -0.23, *P* = 0.18) (Table 4). When the effect of Z*allele on males and females was tested separately, the term habitat quality remained in the best model (Table 4) describing lay date. In one of the sexes the CHD1-Z* genotype had an almost significant statistical effect on lay date (males: t_{353} = -0.48, *P* >0.5, females: t_{422} = -1.93, *P* = 0.05). Males and females started breeding earlier on herb-rich agricultural land than in intensively managed meadows (males: t_8 = -3.28, *P* = 0.01, females: t_{21} = -3.23, *P* = 0.004).

Table 5. Model results of the general linear mixed model of CHD1-Z genotype related to Black-tailed Godwit chick body mass (hatching date is measured as day since 1st of April) on the right and to Black-tailed Godwit chick return rate on the left. Terms left in the model are shown in bold/italic, and the terms are shown in reversed order of exclusion from the model. Estimates and statistics were derived from the last model before exclusion. Reference categories: [1] Z allele, [2] female,[3] 2008, [4] intensively agriculturally managed.

	Estimate	SE	z-value	P		Estimate	SE	t-value	df	P
Return Rates					**Chick Body Mass**					
Intercept	***-3.28***	***0.37***	***-8.88***	***<0.001***	***Intercept***	***28.17***	***1.96***	***14.67***	***803***	***<0.001***
Z[1]	***1.08***	***0.37***	***2.93***	***0.003***	***Z[1]***	***13.08***	***4.14***	***3.16***	***803***	***0.002***
Habitat quality[4]	***-1.36***	***0.44***	***-3.07***	***0.002***	***Z* log(hatching date)***	***-3.49***	***1.089***	***-3.22***	***803***	***0.001***
Year[3]					***log(hatching date)***	***-0.10***	***0.50***	***-0.19***	***803***	***>0.5***
2009	***0.66***	***0.45***	***1.47***	***0.14***	Sex[2]	-0.14	0.09	-1.51	802	0.13
2010	***0.92***	***0.41***	***2.26***	***0.02***	Sex*Z	0.01	0.38	0.02	800	>0.5
Body mass	0.00	0.06	0.01	>0.5	Habitat quality[4]	-0.09	0.21	-0.46	801	>0.5
Z* Body mass	-0.26	0.17	-1.57	0.12						
Z*Habitat quality	1.30	0.96	1.36	0.18	**Random effects**					
Hatching date	-0.01	0.02	-0.61	>0.5	$\sigma 2_{year}$	***0.26***				
Z*Hatching date	0.03	0.04	0.86	0.4	$\sigma 2_{individual}$	***1.99***				
					$\sigma 2_{residuals}$	***1.44***				

Chicks with a Z* genotype had a higher return rate than chicks with a Z genotype (*z* = 2.93, *P* = 0.003). Additionally, chicks that had hatched on intensively managed meadows had a lower return rate (*z* = -3.07, *P* = 0.002). Also the year of hatching influenced return rates of the chicks in consecutive years

(2009: $z = 1.47$, $P = 0.14$, 2010: $z = 2.26$, $P = 0.02$). Hatching date and the interactions of the terms genotype x habitat quality and genotype x hatching date did not remain in the model (Table 5). We had information on body mass of 1470 chicks from 664 nests, of which 129 had a Z* allele. Chicks carrying the Z* allele were heavier if they had hatched early in the season, and lighter if they had hatched late in the breeding season compared with chicks without the Z* allele (Table 5 and Fig. 1). At the mean hatching date of 17 May, chicks were equally heavy.

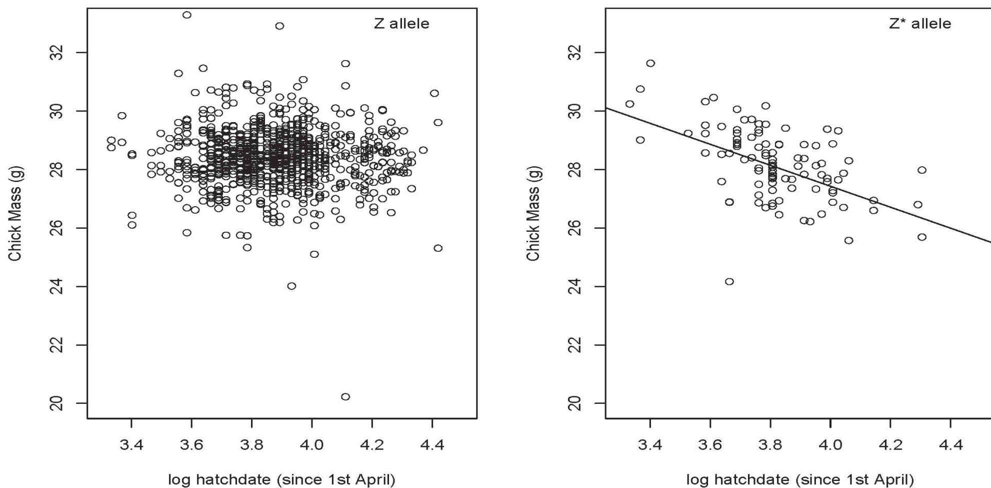


Figure 1. Relationship between hatchdate and chick mass for chicks witout (left) and with the Z* allele (right). The data shown here is corrected for random effects.

Discussion

As pointed out by Benedict *et al.* (2010), links between fitness components and supposedly neutral variation on the sex-chromosome are both surprising and very interesting. Although there were clear correlations between the occurrence of the Z* allele with apparent chick fitness correlates, on the basis of a strongly enlarged sample size we could not confirm all the correlations between Z-allele variants and fitness components in the adults as reported by Schroeder *et al.* (2010).

Our data indicate that the polymorphism does not reflect underlying population structure, a suggestion consistent with a recent study that showed no population structure between Black-tailed Godwits breeding in different parts of The Netherlands (Trimbos *et al.* 2011). Genotype numbers were not significantly different from Hardy-Weinberg or sex ratio expectations, indicating that the

heterozygous genotype did not introduce a selective advantage or disadvantage as compared to the other genotypes. Only one homozygous Z* individual was found, and the biometrics of this chick fell within the population range. Note that on the basis of the rarity of the Z* allele only 4 individuals were expected in the dataset used here.

The fitness calculations in the adults showed one significant, according to the 5% significance criterion, (negative) correlation, between the occurrence of Z* and lay date. Neither adult males nor females carrying the Z* allele showed a higher survival rate, had larger eggs and had a higher body mass or condition than birds without the Z* allele. Schroeder *et al.* (2010) indicated an effect of Z* females breeding earlier than females without the Z* allele (effect size = -4.38 ± 2.15, $P = 0.04$). However, in their analysis they corrected for year only, while we additionally corrected for habitat quality and found a slight effect of Z* on lay date which was almost significant, next to a strong effect of habitat quality. This suggests that the reported correlation in Schroeder *et al.* (2010) may have been partially explained by habitat quality, but also that there might be a tendency that females carrying the Z* allele lay their clutch earlier.

Additionally, we found apparent fitness correlates between the presence of the Z* allele with chick body mass and with chick return rate. The Z* allele had a positive effect on body mass of chicks that had hatched early in the breeding season and a negative effect on chicks that hatched later in the breeding season, compared to chicks without the Z* allele. It is often suggested that early-hatching is related to a higher chick body mass (Arnold *et al.* 2006, Schekkerman *et al.* 2008; 2009, Schroeder *et al.* 2012), and both hatch date and chick body mass often correlate positively with chick survival and should therefore positively influence chick return rates (Arnold *et al.* 2006, Schekkerman *et al.* 2008; 2009, Schroeder *et al.* 2012, Kentie *et al*. 2013). However, we did not find a correlation of chick body mass nor hatching date with return rates, whilst chicks without the Z* allele, the majority of the chicks, did not show a correlation of hatching date with body mass. Return rates were, however, higher for chicks with the Z* allele. Interestingly, our data indicate that birds breeding on herb-rich agricultural land started breeding earlier than birds breeding on intensively managed meadows. Additionally, return rates of chicks that had hatched on herb-rich agricultural land were higher than return rates of chicks that had hatched on intensively managed meadows. This reflects the importance of extensive grassland management for the survival of Black-tailed Godwit chicks, which is the factor responsible for the ongoing annual decline in Black-tailed Godwit breeding populations in northwestern Europe (Teunissen and Soldaat 2005, Schekkerman and Beintema 2007, Schekkerman *et al.* 2008, Zwarts *et al.* 2009).

Whilst not all previous reported associations of Z* with fitness correlates presented by Schroeder *et al.* (2010) could be confirmed here, we did find that Z* females laid earlier and laid eggs which, when hatching a chick with the Z*allele, produced chicks that were also heavier and survived better compared with individuals without the Z* allele. Moreover, given the strong correlations between Z* and fitness in chicks, combined with the notion that Z* chicks have at least one Z* parent, the lay date pattern found in adult females is likely to be a biological reality. Taken together, the results presented here and by Schroeder *et al.* 2010 indeed indicate an association of the Z* allele with fitness.

Under positive selection, the allele frequency of the allele(s) expressing this intronic (and therefore supposedly neutral) variation should become higher through time. Schroeder *et al.* (2010) demonstrated that the Z* allele was present in European Black-tailed Godwits 80 years ago. Although the frequency of the Z* allele could have increased since that time, at present the frequency of the Z* allele is still low. Possibly, because of the weak associations between the Z* allele and the fitness benefits this frequency has not increased noticeably over time. Another explanation might be found through a historical line of reasoning. In response to agricultural intensification, the recruitment of Black-tailed Godwit chicks has been decreasing steadily after the 1960s, resulting in population declines (Schekkerman *et al.* 2008). Between 1900 and the 1960s Black-tailed Godwits were thriving on wet herb-rich grasslands created for dairy farming in northwestern Europe (Haverschmidt 1963, Beintema *et al.* 1995). During this time, due to high chick recruitment, variance of fitness was low throughout the population. As a result the presence of Z*, and the positive effects it has on fitness, probably did not have a selective advantage then. With chick recruitment decreasing since 1967, selective pressure of the Z* allele on chick body mass and return rate might have a more prominent positive effect at present. If so, Z* allele frequencies might still be relatively low because positive selection for this allele only started to become effective recently.

Acknowledgements

We thank It Fryske Gea, the Friese Fûgelwachten, Staatsbosbeheer, Pedro Lourenço, Niko Groen, Petra de Goeij, João Guilherme, Anneke Rippen, Job ten Horn, Robbie Watt, Valentijn van den Brink, Ysbrand Galama, Sytse-Jan Wouda, Rinkje van der Zee, Martin Bulla, Lucie Schmaltz and the students from the University of Groningen for their help in the field and the collection of blood samples. Special thanks goes out to all the farmers for giving us access to their private lands and to the voluntary nest protectors for their most important help in localizing the Black-tailed Godwit nests in the field. Klaas Vrieling and Rene Glas of the Leiden Institute of Biology (IBL) were helpful with PCR amplifications and providing laboratory space in Leiden. Bram Verheijen was of great help with DNA extractions and PCR amplifications of the Black-tailed Godwit blood samples. We were financially supported by a start-up grant to TP from the University of Groningen, a grant to Julia Schoeder from the Schure-Bijerinck-Popping Foundation, a grant by the Vogelbescherming-Nederland and financial support by Province of Fryslân and the Weidevogel Kenniskring of the Ministry of LNV. The work done here was conducted in compliance with the current laws of the country.

References

Agate RJ, Choe M, Arnold AP (2004) Sex differences in structure and expression of the sex chromosome genes CHD1Z and CHD1W in zebra finches. Molecular Biology and Evolution, 21, 384-396.

Arnold JM, Hatch JJ, Nisbet ICT (2006) Effects of egg size, parental quality and hatch-date on growth and survival of Common Tern *Sterna hirundo* chicks. Ibis, 148, 98-105.

Beintema A, Moedt O, Ellinger D (1995) Ecologische Atlas van de Nederlandse Weidevogels. Schuyt & Co, Haarlem.

Benedict L, Bowie RCK, Fuchs J, Macmanes M (2010) When non-coding is non-neutral: the role of CHD1 gene polymorphism in sexing, in phylogenetics and as a correlate of fitness in birds. Ibis, 152, 223-225.

Burnham KP, Anderson DR (2002) Model Selection and Multimodel Inference. New York: Springer, USA.

Casey AE, Jones KL, Sandercock BK, Wisely SM (2009) Heteroduplex molecules cause sexing errors in a standard molecular protocol for avian sexing. Molecular Ecology Resources, 9, 61-65.

Choquet R, Lebreton J, Gimenez O, Reboulet A, Pradel R (2009) U-CARE: Utilities for performing goodness of fit tests and manipulating CApture-REcapture data. Ecography, 32, 1071-1074.

Dawson DA, Darby S, Hunter FM, Krupa AP, Jones IL, Burke T (2001) A critique of avian CHD-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets. Molecular Ecology Notes, 1, 201-204.

Ellrich H, Salewski V, Fiedler W (2010) Morphological sexing of passerines: not valid over larger geographical scales. Journal of Ornithology, 151, 449-458.

Frankham R, Ballou JD, Briscoe DA (2009) Introduction to Conservation Genetics. Cambridge University Press, Cambridge.

Griffiths R, Daan S, Dijkstra C (1996) Sex identification in birds using two CHD genes. Proceedings of the Royal Society B, 263, 1251-1256.

Griffiths R, Double MC, Orr K, Dawson RJG (1998) A DNA test to sex most birds. Molecular Ecology, 7, 1071-1075.

Groen NM, Kentie R, de Goeij P, Verheijen B, Hooijmeijer JCEW, Piersma T (2012) A modern landscape ecology of Black-tailed Godwits: habitat selection in southwest Friesland (The Netherlands). Ardea, 100, 19-28.

Haverschmidt F (1963) The Black-tailed Godwit. E.J. Brill. Leiden

Jarvi SI, Farias MEM (2006) Molecular sexing and sources of CHD1-Z/W sequence variation in Hawaiian birds. Molecular Ecology Notes, 6, 1003-1005.

Kentie R, Hooijmeijer JCEW, Trimbos KB, Groen NM, Piersma T (2013) Intensified agricultural use of grasslands reduces growth and survival of precocial shorebird chicks. Journal of Applied Ecology, 50, 243-251.

Kruk M, Noordervliet MAW, ter Keurs WJ (1997) Survival of black-tailed godwit chicks *Limosa limosa* in intensively exploited grassland Areas in the Netherlands. Biological Conservation, 80, 127-133.

Lee PLM, Brain PF, Forman DW, Bradbury RB, Griffiths R (2002) Sex and death: CHD1Z associated with high mortality in moorhens. Evolution, 56, 2548-2553.

Liebezeit JR, Smith PA, Lanctot RB, Schekkerman H, Tulp I, Kendall DM, Tracy DM, Rodriques RJ, Meltofte H, Robinson JA, Gratto-Trevor C, McCaffery BJ, Morse J, Zack SW (2007) Assessing the development of shorebird eggs using the flotation method: species-specific and generalized regression models. Condor, 109, 32-47.

Lourenço PM, Kentie R, Schroeder J, Groen NM, Hooijmeijer JCEW, Piersma T (2011) Repeatable timing of northward departure, arrival and breeding in Black-tailed Godwits *Limosa l. limosa*, but no domino effects. Journal of Ornithology, 152, 1023-1032.

Montel H, Fridolfsson A, Ellegren H (2001) Contrasting levels of nucleotide diversity on the avian Z and W sex

chromosomes. Molecular Biology and Evolution, 18, 2010-2016.

Richardson DS, Jury FL, Blaakmeer K, Komdeur J, Burke T (2001) Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). Molecular Ecology, 10, 2263-2273.

Saino N, Rubolini D, Serra L, Caprioli M, Morganti M, Ambrosini R, Spina F (2010) Sex-related variation in migration phenology in relation to sexual dimorphism: a test of competing hypotheses for the evolution of protandry. Journal of Evolutionary Biology, 23, 2054-2065.

Schekkerman H, Beintema AJ (2007) Abundance of invertebrates and foraging success of Black-tailed Godwit *Limosa limosa* chicks in relation to agricultural grassland management. Ardea, 95, 39-54.

Schekkerman H, Teunissen WA, Oosterveld E (2009) Mortality of Black-tailed Godwit *Limosa limosa* and Northern Lapwing *Vanallus vanellus* chicks in wet grasslands: influence of predation and agriculture. Journal of Ornithology, 150, 133-145.

Schekkerman H, Teunissen WA, Oosterveld E (2008) The effect of 'mosaic management' on the demography of black-tailed godwit *Limosa limosa* on farmland. Journal of Applied Ecology, 45, 1067-1075.

Schroeder J, Kentie R, van der Velde M, Hooijmeijer JCEW, Both C, Haddrath O, Baker AJ, Piersma T (2010) Linking intronic polymorphism on the CHD1-Z gene with fitness correlates in Black-tailed Godwits *Limosa l. limosa*. Ibis, 152, 368-377.

Schroeder J, Piersma T, Groen NM, Hooijmeijer JCEW, Kentie R, Lourenço PM, Schekkerman H, Both C (2012) Reproductive timing and investment in relation to spring warming and advancing agricultural schedules. Journal of Ornithology, 153, 327-336.

Stokes DG, Perry RP (1995) The DNA-binding and chromatin-localization properties of CHD1. Molecular and Cellular Biology, 15, 2745-2753.

Teunissen W, Soldaat L (2005) Indexen en trends van een aantal weidevogelsoorten uit het Weidevogelmeetnet. Periode 1990-2004. SOVON-informatie 2005/13 SOVON Vogelonderzoek Nederland Beek-Ubbergen.

Toms JD, Eggert LS, Wayne JA, Faaborg J (2012) A genetic polymorphism in the sex-linked ATP5A1 gene is associated with individual fitness in Ovenbirds (*Seiurus aurocapilla*). Ecology & Evolution, 2, 1312-1318.

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.

Trimbos KB, Musters CJM, Verkuil IV, Kentie R, Piersma T, de Snoo GR (2011) No evident spatial genetic structuring in the rapidly declining Black-tailed Godwit *Limosa limosa limosa* in The Netherlands. Conservation Genetics, 12, 629-636.

van der Meer J, Piersma T (1994) Physiologically inspired regression models for estimating and predicting nutrient stores and their composition in birds. Physiological Zoology, 67, 305-329.

White GC, Burnham KP (1999) Program MARK: survival estimation from populations of marked animals. Bird Study, 46, S120-S139.

Zwarts L, Bijlsma RG, van der Kamp J, Wymenga E (2009) Living on the edge. Wetlands and Birds in a Changing Sahel. KNNV Publishing, Zeist.

CHAPTER

4

No evident spatial genetic structuring in the rapidly declining Dutch Black-tailed Godwit *Limosa l. limosa* population

Conservation Genetics (2011), 12, 629-636

Krijn B. Trimbos, Cees J. M. Musters, Yvonne Verkuil, Rosemarie Kentie,
Theunis Piersma, Geert R. de Snoo

Abstract

With 40% of the European Black-tailed Godwit population breeding in The Netherlands, this country internationally harbours important numbers of this species. However, ongoing agricultural intensification has resulted in the fragmentation of the population and drastic population declines since 1967. Establishing genetic diversity, genetic differentiation and gene flow on the basis of 12 microsatellites, we investigated whether the population genetic structure of the Dutch Black-tailed Godwit bears the marks of these changes. Genetic diversity appeared moderate and Bayesian model-based analysis of individual genotypes revealed no clustering in the Dutch populations. This was supported by pairwise F_{ST} values and AMOVA, which indicated no differentiation among the nine separate breeding areas. Gene flow estimates were larger than “one migrant per generation” between sample locations, and no isolation by distance effect was demonstrated. Our results indicate the maintenance of moderate levels of genetic diversity throughout the Dutch Black-tailed Godwit population through appropriate levels of gene flow between different breeding areas. We suggest that the Dutch Black-tailed Godwit breeding areas should be managed as a single panmictic unit, much as it is presently done.

Keywords Genetic differentiation · Genetic diversity · Gene Flow · Habitat fragmentation · Microsatellites · Negative population trends

Introduction

For a long time agriculture in Europe generated high bird species diversity, but with increasing intensification over the last 50 years, many species now show seriously negative trends (Chamberlain *et al.* 2000, Stoate *et al.* 2009, Teunissen and Soldaat 2005, van Turnhout *et al.* 2007). A good example of a bird species that waxed and waned in response to agricultural land use changes is the Black-tailed Godwit *Limosa limosa limosa* (Bijlsma *et al.* 2001, Birdlife International 2009). The Black-tailed Godwit was previously confined to raised bogs, moorlands, lake margins and damp grassy depressions in steppe. However when wet grassland created for the purpose of dairy farming increased in northwestern Europe, this species became very successful through exploitation of this habitat (Beintema *et al.* 1995, Haverschmidt 1963). Close to half the European Black-tailed Godwit population was known to breed in The Netherlands (Birdlife International 2004, Teunissen and Soldaat 2005). However, continuing declines of 5% per year since the peak numbers of the late 1970s (Schroeder *et al.* 2009) have decreased Black-tailed Godwit breeding numbers. While 120,000 pairs (Mulder 1972) were estimated to breed in 1967, only 40,000 pairs remained in 2004 (Teunissen and Soldaat 2005).

The most significant threats for this species include loss of nesting habitat owing to wetland drainage and agricultural intensification. The earliest modernization of farming enhanced food supply and thus increased population sizes of several wader species (Cramp and Simons 1983). However, further intensification practices have resulted in reduced food availability, lower water tables, increased cattle densities, and increased early mowing (Benton 2001, Bijlsma *et al.* 2001, Schekkerman *et al.* 2008). Furthermore, predation risk has increased as a result of early mowing practices, mostly due to reduced coverage for nesting, and chick raising (Schekkerman *et al.* 2009). These agricultural adjustments have in turn culminated in impaired chick recruitment and decreasing habitat quality. Subsequently, the declining habitat quality has led to the fragmentation of suitable grassland (Teunissen and Soldaat 2005, Zwarts *et al.* 2009). Schekkerman *et al.* (2008) documented a decline from 0.9 fledged chicks per godwit pair in 1985 to roughly 0.23 fledged chicks per pair in 2006.

Although, Black-tailed Godwit habitat is becoming more fragmented and habitat quality decreasing, this species shows high breeding site fidelity and some natal philopatry (Groen 1993, van den Brink *et al.* 2008). Groen (1993) showed 90% of the adult breeding birds returned within 700 m of the previous nest site. These results are supported by van den Brink *et al.* (2008) who found that 100% of the adult Black-tailed Godwits returned within 3 km of the former nest site. Natal philopatry was demonstrated to be high as well with 75% of the birds returning within 18 km of their previous hatching site. With adult dispersal being limited and the number of chicks dispersing beyond 18 km theoretically decreasing due to declining recruitment, breeding sites could have become partly or completely isolated from each other. This might affect population dynamics, resulting in a metapopulation structure including source-sinks or isolation by distance (Höglund 2009).

Genetic connectivity between areas is maintained by dispersal of successfully reproducing animals among breeding areas, i.e. gene flow. Slatkin (1985, 1987) concluded that only one migrant per generation is needed to obscure any disruptive effects of genetic drift. On the other hand, Mills and

Allendorf (1996) suggest that this number should actually be larger than 1 in many natural populations and that the one migrant per generation rule should be considered as a minimum. Additionally, another study showed that the size of the recipient population(s) under study might also influence the number of migrants needed to avoid excessive inbreeding (Vucetich and Waite 2000).

Here we studied gene flow indirectly by investigating genetic diversity and differentiation on a national scale covering the most important breeding areas of the Dutch Black-tailed Godwit breeding population (Figure 1). 12 microsatellite loci developed specifically for this species by Verkuil *et al.* (2009) were used. First, the markers were validated to evaluate if they were polymorphic and were in congruence with the assumptions made by several population genetic software. Second, the population genetic structure of the Dutch Black-tailed Godwit breeding sites was assessed through genetic diversity, genetic differentiation and gene flow calculations. Moreover, if genetic structure (isolation of breeding areas with genetic differentiation between areas and or low genetic diversity) or the lack of it was portrayed, we tried to explain the underlying mechanism.

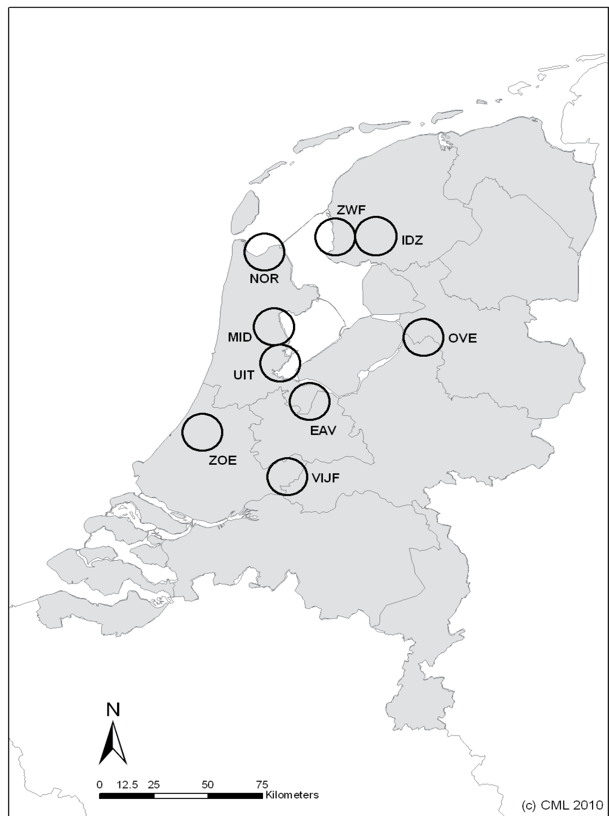


Figure 1. Sample locations; Zuid-West Fryslân (ZWF), Eemnes/Arkemheen/Vinkeveen (EAV), Zoeterwoude (ZOE), Idzegea (IDZ), Middelie (MID), Normerpolder (NOR), Vijfheerenlanden (VIJF), Uitdam (UIT) and OVE (Overijssel).

Material and methods

Sample collection, DNA extraction and amplification

From 2004 to 2008, blood samples from freshly hatched chicks and dry egg shell remains (Trimbos *et al.* 2009) were collected in nine distinct regions in The Netherlands (Figure 1). Regions were from 7 to 135 km apart. With the help of voluntary nest protectors, nests were located early during incubation, through continuous observational effort. When a nest was found, the floating method of Liebezeit *et al.* (2007) was used to determine hatching date. Around the hatching date the nests were visited daily to obtain either eggshells or ca. 30 µl whole blood per chick. To minimize post-sampling contamination, individual egg shells were stored in plastic bags. Blood was stored in individual 1.5 ml Eppendorf tubes containing 97% alcohol buffer. Blood samples were stored at -70 °C, while egg shells were stored at room temperature to get a good separation of the egg shell membranes from the outer shell. DNA samples of 140 individual Black-tailed Godwits were collected (Table 1). These 140 samples incorporated only one individual per nest to keep relatedness between samples as low as possible.

DNA was extracted from 6-10 µl of blood using the Ammonium Acetate method as described by Richardson *et al.* (2001). DNA was extracted from eggshell membrane using Qiagen Dneasy Tissue Kit (Qiagen 2003), with minor modifications as described by Trimbos *et al.* (2009). DNA quality and quantity were checked twice, using the NanoDrop ND-1000 (Thermo Scientific) for 260/280 ratios and concentration values. For optimal PCR amplification, blood samples were diluted to concentrations below 10 ng/µl. Compared to blood derived DNA, DNA from eggshell membranes was of less purity occasionally. Consequently, eggshell derived DNA was diluted to concentrations below 50 ng/µl. We used 12 microsatellite loci (LIM3, LIM5, LIM8, LIM10, LIM11, LIM12a, LIM24, LIM25, LIM26, LIM30, LIM33) developed for Black-tailed Godwits (Verkuil *et al.* 2009). The final volumes of the PCR amplification mix were 11 µl and included 1-10 ng DNA for blood samples or 1-50 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, 10x PCR buffer and 0.45 U Taq DNA Qiagen polymerase. The polymerase chain reaction program used was as described by Verkuil *et al.* (2009), except the final PCR step was extended to 20 min to minimize peak stutter patterns. PCR products were analyzed using a MegaBACE 1000 (Amersham Biosciences) and allele sizes were assigned using Fragment Profiler 1.2 (Amersham Biosciences 2003). Contamination of PCR pre-mix with exogenous DNA was minimized by carrying out pre- and post-PCR pipetting in different rooms. Additionally, to control for potential contamination problems, negative controls were included in every PCR reaction and MegaBACE runs.

Genetic marker validation

Microsatellite markers are expected to be independently distributed in the genome as such linkage between loci would result in pseudo-replication (Selkoe *et al.* 2006). A Fisher's exact test for linkage disequilibrium was carried out using the samples from the nine breeding areas, with 1,000 dememorization steps, 100 batches and 1,000 iterations per batch (GENEPOP; Raymond and Rousset 1995, web version 4.0). Deviations from Hardy-Weinberg, heterozygote excess and deficit were tested per

locus and sample location separately using 1,000 dememorization steps, 100 batches and 1,000 iterations per batch (GENEPOP; Raymond and Rousset 1995). For multiple testing Bonferroni correction was applied (Rice 1989). MICRO-CHECKER was used to test for scoring and amplification errors (stutter and null alleles) with a 95% confidence interval over 10,000 runs (Oosterhout *et al.* 2004). To evaluate genotyping error, scoring was performed three times and the frequency of disagreement between different times of scoring was noted and averaged.

Genetic diversity, F_{IS} and population structure analyses

Observed (H_o), expected heterozygosity (H_e), inbreeding values (F_{IS}) per location and pairwise F_{ST} between locations were calculated using ARLEQUIN 3.11 (Excoffier *et al.* 2005). Furthermore, an analyses of molecular variance (AMOVA) was performed, through which variance among sample locations (V_a), among individuals within sample locations (V_b), and within all individuals could be computed (V_c), using ARLEQUIN with 20,000 permutations. If significant values were obtained, Bonferroni correction was applied. Number of private alleles was determined using CONVERT 1.31 (Glaubitz 2004). FSTAT 2.9.3.2 (Goudet 1995) was used to calculate allelic range, number of alleles per sample location and allelic richness per sample location. This program uses the rarefaction index, as described by Hurlbert (1971), to correct for sample size. Additionally, the levels of allelic richness and F_{IS} among sample locations were compared using FSTAT with 10,000 permutations to obtain P-values.

The model based Bayesian cluster algorithm implemented in STRUCTURE 2.3.1 (Pritchard *et al.* 2000) was used to cluster from a pool of genotypes from all sampling locations. We determined the deltaK (Structure Harvester) which is a calculation of the second order rate of change in log likelihood Ln P(X|K) recommended by Evanno *et al.* (2005). Although this method was demonstrated to be more reliable in estimating the inferred amount of clusters in natural populations, K = 1 cannot be measured which was a reasonable possibility for this research. Consequently, the most likely number of genetic clusters (K) in our sample set was also investigated by determining the maximum average log likelihood Ln P(X|K). Values computed with both methods were plotted using Structure Harvester 0.56.3 (Dent 2009, web version). The Structure model was run using admixture and correlated allele frequencies. Additionally, the LOCPRIOR model, incorporated into STRUCTURE 2.3.1, was used. This model assumes that individuals sampled close together are often from the same population and can assist in the clustering when population structure is weak. The program was run 5 times with a burn-in period of 200,000 iterations and a length of 1,000,000 MCMC iterations for K (1-11). Convergence was checked by looking whether the graphs provided by the program reached equilibrium before the end of the burn-in phase.

The genetic structure profile within this dataset could display a historic situation of Black-tailed Godwit population dynamics. The Dutch Black-tailed Godwit breeding population is believed to have expanded from 1900 until the 1960s. The k test as implemented in the program Kgtests (Bilgin 2007) detects population expansion on the basis of allele size distributions. The method uses a one tailed binomial distribution to test for the number of loci with negative k values and if this represents a significant number of negative k values. Additionally, this software included the g test, which tests the notion that stable populations are reflected by highly variable variances of allele sizes among

loci, while in an expanding population this variance is more equal. Both tests were preformed here, although Luikart *et al.* (1998) demonstrated that the g test was the more powerful of the two.

Gene flow patterns between sample locations

The number of migrants between the different sample locations was estimated using Slatkin's (1985) private allele method which is incorporated in the GENEPOP 4.0 (Raymond and Rousset 1995). This calculation assumes an approximately equilibrium distribution of allele frequencies among the demes comprising a population (Barton and Slatkin 1986). Most coalescent computer programs, developed to calculate gene flow between populations and effective population size, assume stable (sub) populations over time (Kuhner 2008). As the Dutch Black-tailed Godwit population is believed to have been rather variable in size over the last 100 years (Beintema *et al.* 1995, Schekkerman *et al.* 2008, Schroeder *et al.* 2009), we refrained from using these programs. Nevertheless, there are programs such as IMa2 (Hey and Nielson 2007) that allow testing of migration rates between different locations in populations with a probably unstable subpopulation structure over time. However, this program uses a tree string as a backbone to make coalescent inferences. Unfortunately, there were several uncertainties in our data in constructing such a tree correctly and as such this program was not used further. To explore dispersal limitation issues due to the confounding effect of geographic distance a Mantel test (normally transformed and log transformed) with 9999 permutations was preformed using GENALEX 6.2 (Peakall and Smouse 2006) which calculates the correlation between a genetic and a geographic distance matrix (Smouse and Long 1992, Smouse *et al.* 1986).

Results

Genetic marker validation

A total of 140 birds from 9 different breeding locations were genotyped. All 12 loci amplified no more that two alleles per individual. All loci were polymorphic with 4 to 15 alleles per locus. We detected 126 different alleles.

Some loci in some locations exhibited significant deviations from Hardy-Weinberg. However, none of these values remained significantly different from zero after sequential Bonferroni correction. After Bonferroni correction no linkage-disequilibrium was found between any of the loci in any of the locations. The mean genotyping error, the averaged difference between the 1st and 2nd and 1st and 3rd time of scoring, was 1.5%. MICRO-CHECKER showed no presence of null alleles at any of the sample locations or loci.

Genetic diversity and F_{IS} and population structure analysis

The mean number of alleles, absolute number of alleles, allelic richness, Ho, He, F_{IS}, and private alleles, per sample location are assembled in Table 1. There was no relationship between genetic diversity values (Table 1) and sample location. F_{IS} was not significantly different from zero in any location. Subsequently, the differences in allelic richness (N =9, P = 0.079) and F_{IS} (N = 9, P = 0.866) among sample locations were not significant.

Table 1. Number of samples (N), mean number of alleles (N_a), absolute number of alleles (A), allelic richness (A_R), Observed heterozygosity (H_o), Expected heterozygosity (H_e), inbreeding coefficient (F_{IS}) and number of private alleles (P_a), per sample location (Figure 1) using 12 microsatellites.

Sample location	N	N_a	A	A_R	Ho	He	F_{IS}	P_a
ZWF	38	8	96	5.134	0.686	0.682	-0.024	5
EAV	24	7.5	90	4.987	0.628	0.674	0.053	4
ZOE	11	6	72	5.133	0.697	0.689	-0.012	1
IDZ	18	7.1	85	5.259	0.685	0.689	0.005	1
MID	11	5.8	70	5.094	0.694	0.695	-0.018	1
NOR	7	5.3	64	5.333	0.715	0.722	0.011	1
VIJF	10	6.2	74	5.351	0.683	0.679	-0.006	1
UIT	11	5.8	69	4.944	0.616	0.647	0.002	0
OVE	10	5.4	65	4.794	0.643	0.689	0.050	0
Average	15.0	6.3	76.0	5.115	0.671	0.685	0.007	1.6

Nearly all pairwise F_{ST} values between locations were not significantly different from zero, except for those between ZWF and VIJF (Table 2). However, after Bonferroni correction this significance did not hold. AMOVA calculations showed no significance for any of the calculated variances (0% V_a = -0.0011 P = 1.000 ± 0.000, 0.3% V_b = 0.004 P = 0.37793 ± 0.00353 and 99.7% V_c= 0.004 P = 0.37903 ± 0.00358).

Structure analyses indicated that the most likely value for the amount of genetic clusters (K) was K = 1. Using the method as described by Evanno *et al.* (2005) and plotting delta K did not result in a 'plateau' and as such it was not clear what value for K was the most likely. Maximum average log likelihood Ln P(P|K) values plotted against number of inferred clusters (K) demonstrated that K = 1 best fit the data (Figure 2), as the highest log likelihood was obtained with K = 1.

Table 2. Pairwise F_{ST}, on the left side of the table, and according P values, on the right side of the table, are given. P values smaller than 0.05, are indicated with *.

	ZWF	EAV	ZOE	IDZ	MID	NOR	VIJF	UIT	OVE
ZWF		0.085±0.002	0.592±0.003	0.545±0.003	0.709±0.003	0.348±0.003	0.022±0.001*	0.947±0.0015	0.310±0.003
EAV	0.005		0.691±0.003	0.252±0.003	0.451±0.004	0.743±0.003	0.051±0.002	0.607±0.003	0.278±0.000
ZOE	-0.002	-0.003		0.554±0.004	0.901±0.002	0.525±0.003	0.173±0.003	0.849±0.003	0.630±0.003
IDZ	-0.001	0.004	-0.002		0.528±0.003	0.381±0.004	0.308±0.003	0.973±0.001	0.453±0.004
MID	-0.004	0.001	-0.011	-0.001		0.684±0.003	0.961±0.001	0.959±0.001	0.504±0.004
NOR	0.002	-0.005	-0.002	0.003	-0.007		0.814±0.003	0.142±0.002	0.670±0.003
VIJF	0.013	0.015	0.008	0.003	-0.015	-0.010		0.528±0.003	0.329±0.004
UIT	-0.009	-0.001	-0.010	-0.013	-0.017	0.013	-0.001		0.885±0.002
OVE	0.003	0.006	-0.004	0.001	0.000	-0.004	0.005	-0.011	

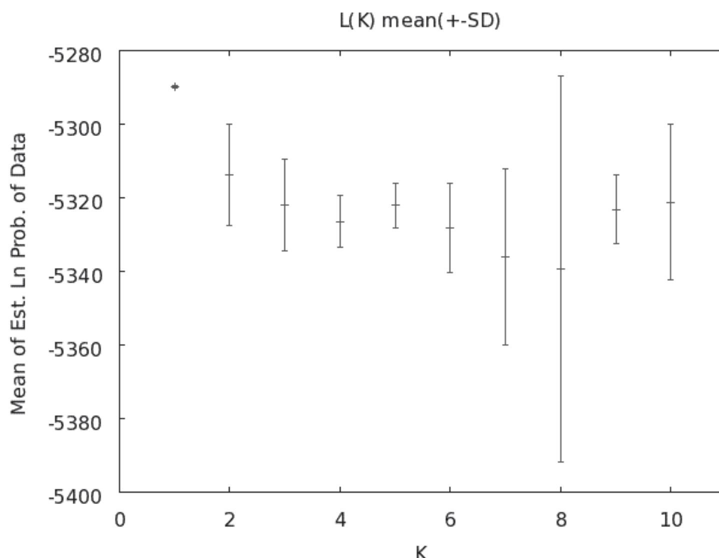


Figure 2. Mean log likelihood Ln P(X|K) as a function of the number of genetic clusters (K) averaged over 5 consecutive STRUCTURE runs for each K (error bars indicate one standard deviation).

Kgtests (Banlin 2007) indicated no population expansion in the dataset. The k test revealed that 9 out of 12 loci had a negative kurtosis which was almost significant (P = 0.06). The g test estimated that the variance of the allele sizes was 8.01. According to the fifth-percentile cutoff table given by Reich *et al.* (1999) this value was not significant.

Gene flow patterns between sample locations

Mantel tests detected no significant correlation between genetic distance and either measure of geographic distance (P = 0.157 normally transformed and log transformed P = 0.448). The number of migrants (Nm) per generation (Table 3) showed a range from 4.96 (between ZWF and EAV) to 1.50 (between NOR and OVE). The average number of migrants per generation among the Dutch breeding locations, and corrected for sample size, was 2.79.

Table 3. Number of migrants per generation between 2 sample locations.

	ZWF	EAV	ZOE	IDZ	MID	NOR	VIJF	UIT	OVE
ZWF		4.96	3.93	4.74	3.17	2.35	3.22	3.99	2.66
EAV	4.96		3.41	3.18	3.65	2.36	2.48	4.12	2.49
ZOE	3.93	3.41		2.63	2.28	1.98	1.92	2.68	2.35
IDZ	4.74	3.18	2.63		2.56	2.12	4.10	3.65	2.49
MID	3.17	3.65	2.28	2.56		1.76	2.10	2.50	2.09
NOR	2.35	2.36	1.98	2.12	1.76		1.79	1.82	1.50
VIJF	3.22	2.48	1.92	4.10	2.10	1.79		2.70	2.88
UIT	3.99	4.12	2.68	3.65	2.50	1.82	2.70		1.92
OVE	2.66	2.49	2.35	2.49	2.09	1.50	2.88	1.92	

Discussion

We demonstrated an absence of genetic structure among nine Dutch Black-tailed Godwit breeding locations. Does this lack of genetic structure reflect limited power due to an insufficient number of microsatellites (Barnett *et al.* 2008)? We suggest this not to be the case as several studies have shown genetic structure using seven or even fewer microsatellites (Davis *et al.* 2006, Rönkä *et al.* 2008). Additionally, several studies demonstrated the lack of genetic structure using as many as 13 (Barnett *et al.* 2008) and 29 microsatellite markers (Van Treuren *et al.* 1999). Furthermore, the microsatellites used did not exhibit Hardy-Weinberg and linkage disequilibrium and showed no null allele problem. All the loci used were polymorphic with ranges between 4 and 15 alleles and a total of 126 alleles in the entire dataset. According to Ryman *et al.* (2006) these values of allelic range and total amount of alleles in combination with a sample size of 140 individuals and 12 microsatellites used, should be sufficient to detect genetic structure. As such it seems very unlikely that absence of genetic structure with the Dutch Black-tailed Godwit population can be appointed to insensitivity or amount of the microsatellites used.

Nevertheless, genetic structure can be influenced by the dispersal abilities and the extent of habitat fragmentation of the species under study. The detection of dispersal abilities and habitat fragmentation depend on the scale of the study. As such the detection of genetic structure depends to a large degree on the scale of the study as well (Barnett *et al.* 2008). In studies on other bird species (Temple *et al.* 2006, Woxvold *et al.* 2006), genetic structure was evident using only six microsatellites on much smaller scales (longest distance between locations 4 km and 8 km) compared to the spatial scale (a maximum of 134 km between sites) in this study. This demonstrates that even on very small spatial scales, in some birds, genetic structure can be detected. Obviously, this depends on the birdspecies under study as well as the landscape it lives in.

The multi-locus microsatellite data presented here suggest the most likely explanation for the lack of genetic structure in the Dutch Black-tailed Godwit is that breeding areas in The Netherlands comprise one panmictic unit. Gene flow estimates demonstrated an overall migration rate of three individuals per generation among Dutch breeding locations. According to both Mills and Allendorf (1996) and Slatkin (1985) this rate should be enough to minimize genetic differentiation. Subsequently, results of the Mantel test demonstrated no isolation by distance that would indicate restrictions on gene flow. This shows that dispersal movements have taken place well beyond the 18 km range, as far as 134 km, which demonstrates the high breeding mobility capabilities of this species. Groen (1993) and van den Brink *et al.* (2008) have most likely underestimated the dispersal distance of this species. This is supported by unpublished observations of Kentie *et al.* which indicate that adult birds seek out new breeding sites up to 10 km from the previous breeding site. Still, the most probable explanation for these findings is natal dispersal.

Bayesian analysis in STRUCTURE showed that all individual Black-tailed Godwits could most likely be assigned to one genetic cluster. This was supported by pairwise F_{ST} calculations which demonstrated little (ranging from -0.0168 to 0.01337) or no significant differentiation between locations.

Additionally, none of the F_{IS} values were significantly different from zero for any of the locations, indicating no inbreeding . Subsequently, AMOVA showed that more than 99% of the molecular variation was found across all individuals while an insignificant proportion (0.3%) was attributable to variation between individuals from different locations.

Interestingly, Schroeder *et al.* (2010) showed the presence of a polymorphism in the CHD1-Z gene. The rare polymorphism (Z*, 14% of population) appeared to be associated with fitness advantages and only occurred in Black-tailed Godwit breeding in reserves managed as meadowbird habitat. Although there still is the problem of statistical power (33 individuals sampled outside meadowbird reserves and 251 birds in reserves), the fitness correlates indicated that this genetic marker was non-selectively neutral and thereby not a suitable marker for evaluating neutral population structuring (Schroeder *et al.* 2010). Wolf *et al.* (2010) suggested that markers that are linked to genes under expression evolve much faster than do non-coding genes. This may in turn have resulted in incipient population structuring measurable with the appropriate non-selective neutral marker, whilst it has not lead to it being measurable using neutral markers.

The level of genetic diversity in the Dutch Black-tailed Godwit in this study is higher (average of 6.3 alleles per locus) compared to that reported by Höglund *et al.* (2009) who found rather low genetic diversity (1 haplotype) within the Dutch Black-tailed Godwit (*Limosa limosa limosa*) using mitochondrial DNA. However, the marker used in this study amplifies the second domain of the control region a part that is highly conserved (Höglund *et al.* 2009) and might therefore be less suitable for detection of genetic variation within the Dutch Black-tailed Godwit. The genetic diversity within the Dutch Black-tailed Godwit was therefore likely underestimated in this study. Furthermore, most Dutch samples came from the Western part of The Netherlands while 50% of the population resides in the northern part of the country, possibly holding a significant amount of the total population genetic variation.

Interestingly, using mtDNA from four different Dutch Black-tailed Godwit breeding sites Höglund *et al.* (2009) did not detect any genetic structure either. This result together with the results demonstrated here would indicate that all locations are affected by long term panmixis or that possible gene flow between different breeding areas ceased too recently for both marker types to be detectable (Zink and Barrowclough 2008).

All together it appears that the Dutch Black-tailed Godwit population is not confronted with immediate genetic threats and we argue that according to these data the Dutch Black-tailed Godwit should be managed as one panmictic unit.

Acknowledgements

We hereby declare that the experiments comply with the current laws of the country in which they were performed. Lida Kanters, David Kleijn, Rene Faber, Astrid Kant, Gerrit Gerritsen, Wim Tijssen and Dirk Tanger were all of existential help collecting egg shell membrane samples at several Dutch Black-tailed Godwit breeding locations. We would like to thank Jos Hooijmeijer, Petra de Goeij, Pedro Lourenço and the rest of the Groningen University Black-tailed Godwit group for their help in collecting eggshells and blood samples in South-West Fryslân, sharing their blood samples collected at other Dutch Black-tailed Godwit breeding sites and providing laboratory space for DNA extractions. Marco van der Velde from the University of Groningen was of great help with the DNA extractions from blood samples. Klaas Vrieling and Rene Glas of the Leiden Institute of Biology (IBL) were helpful with PCR amplifications and providing laboratory space. Dick Groenenberg and Camiel Doorenweerd helped with the interpretation of the data.

References

Barnett JR, Ruiz-Gutierrez V, Coulon A, Lovette IJ (2008) Weak genetic structuring indicates ongoing gene flow across White-ruffed Manakin (*Corapipo Altera*) populations in a highly fragmented Costa Rica landscape. Conservation Genetics, 9, 1403-1412.

Barton NH, Slatkin M (1986) A Quasi-equilibrium theory of the distribution of rare alleles in a subdivided population. Heredity, 56, 409-415.

Beintema AJ, Beintema-Hietbrink RJ, Muskens GJDM (1985) A shift in the timing of breeding in meadow birds. Ardea, 73, 83-89.

Beintema A, Moedt O, Ellinger D (1995) Ecologische Atlas van de Nederlandse Weidevogels. Schuyt & Co, Haarlem.

Benton TG, Bryant DM, Cole L, Crick HOP (2002) Linking agricultural practice to insect and birdpopulations: a historical study over three decades. Journal of Applied Ecology, 39, 673-687.

Bijlsma RG, Hustings F, Camphuysen CJ (2001) Algemene en schaarse vogels van Nederland (Avifauna van Nederland 2). Haarlem/Utrecht: GMB Uitgeverij/KNNV Uitgeverij.

Bilgin R (2007) Kgtests: a smiple Excel Macro program to detect signatures of population expansion using microsatellites. Molecular Ecology Notes, 7, 416-417.

BirdLife International (2009) Species factsheet: *Limosa limosa*. Downloaded from http://www.birdlife.org on 29/1/2010.

Birdlife International (2004). Birds in Europe: population estimates, trends and conservation status. Cambridge, UK.

Chamberlain DE, Fuller RJ, Bunce RGH, Duckworth JC, Shrubb M (2000) Changes in the abundance of farmland birds in relation to the timing of agricultural intensification in England and Wales. Journal of Applied Ecology, 37, 771-788.

Davis LA, Roalson EH, Cornell KL, McClanahan KD, Webster MS (2006) Genetic divergence and migration patterns in a North American passerine bird: implications for evolution and conservation. Molecular Ecology, 15, 2141-2152.

Dent EA (2009) STRUCTURE HARVESTER version 0.56.3 http://taylor0.biology.ucla.edu/struct_harvest/.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology, 14, 2611-2620.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, 1, 47-50.

Glabutz JC (2004) CONVERT: a user friendly program to reformat diploid genotypic data for commonly used population genetic software packages. Molecular Ecology Notes, 4, 309-310.

Groen NM (1993) Breeding site tenacity and natal philopatry in the Black-tailed Godwit. Ardea, 81, 107-113.

Goudet J (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. Journal of Heredity, 86, 485-486.

Haverschmidt F (1963) The black-tailed godwit. E.J. Brill. Leiden

Hey J, Nielsen R (2007) Integration within the Felsenstein equation for improved Markov chain Monte Carlo methods in population genetics. PNAS, 104, 2785-2790.

Höglund J, Johansson T, Beintema A, Schekkerman H (2009) Phylogeography of the Black-tailed Godwit *Limosa limosa*: substructuring revealed by mtDNA control region sequences. Journal of Ornithology, 150, 45-53.

Höglund J (2009) Evolutionary Conservation Genetics. Oxford university press, Oxford, New York.

Hurlbert SH (1971) The nonconcept of species diversity: a critique and alternative parameters. Ecology, 52, 577-586.

Kuhner MK (2009) Coalescent genealogy samplers: windows into population history. Trends Ecology and Evolution, 24, 86-92.

Liebezeit JR, Smith PA, Lanctot RB, Schekkerman H, Tulp I, Kendall SJ, Tracy DM, Rodriques RJ, Meltofte H, Robinson JA, Gratto-Trevor C, McCaffery BJ, Morse J, Zack SW (2007) Assessing the development of shorebird eggs using the flotation method: Species-specific and generalized regression models. Condor, 109, 32-47

Luikart G, Sherwin WB, Steele BM, Allendorf FW (1998) Usefulness of molecular markers for detecting population bottlenecks via monitoring genetic change. Molecular Ecology, 7, 963–974.

Mills LS, Allendorf FW (1996) The one-migrant-per-generation rule in conservation and management. Conservation Biology, 10, 1509-1518.

Mulder T (1972) De grutto (*Limosa limosa* (L)) in Nederland: aantallen, verspreiding terreinkeuze, trek en overwintering. Bureau van de KNNV, Hoogwoud.

Peakal R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes, 6, 288-295.

Pritchard JK, Stphens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics, 155, 945-959.

Qiagen (2003) DNeasy Tissue Handbook. Protocol for isolation of total DNA from animal tissues. pp.18-20, QIAGEN. Valencia, California, USA.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity, 86, 248-249.

Reich DE, Feldman MW, Goldstein DB (1999) Statistical properties of two tests that use multilocus data sets to detect population expansions. Molecular Biology and Evolution, 16, 453-466.

Rice WR (1989) Analysing tables of statistical tests. Evolution, 43, 223-225

Richardson DS, Jury FL, Blaakmeer K, Komdeur J, Burke T (2001) Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). Molecular Ecology, 10, 2263-2273.

Rönkä A, Kvist L, Karvonen J, Koivula K, Pakanen VM, Schamel D, Tracy DM (2008) Population genetic structure in the Temminck's stint *Calidris temminckii*, with an emphasis on Fennoscandian populations. Conservation Genetis, 9, 29-37.

Ryman N, Palm S, Andre C, Carvalho GR, Dahlgren TG, Jorde PR, Laikre L, Larsson LC, Palme A, Ruzzante DE (2006) Power for detecting genetic divergence: differences between statistical methods and marker loci. Molecular Ecology, 15, 2031-2045.

Schekkerman H, Teunissen WA, Oosterveld E (2009) Morality of Black-tailed Godwit *Limosa limosa* and Northern Lapwing *Vanallus vanellus* chicks in wet grasslands: influence of predation and agriculture. Journal of Ornithology, 150, 133-145

Schekkerman H, Teunissen WA, Oosterveld E (2008) The effect of 'mosaic management' on the demography of black-tailed godwit *Limosa limosa* on farmland. Journal of Applied Ecology, 45, 1067-1075.

Schröder J, Hinsch M, Hooimeijer JCE, Piersma T (2009). Faillissement dreigt voor Nederlands weidevogelbeleid. De Levende Natuur, 110, 333-338.

Schroeder J, Kentie R, van der Velde M, Hooijmeijer JCEW, Both C, Haddrath O, Baker AJ, Piersma T (2010) Linking intronic polymorphism on the CHD1-Z gene with fitness correlates in Black-tailed Godwits *Limosa l. limosa*. Ibis, 152, 368-377.

Slatkin M (1987) Gene flow and the geographic structure of natural populations. Science, 236, 787-792.

Slatkin M (1985) Gene flow in natural popualtions. Annual review of Ecology and systematic, 16, 393-430.

Smouse PE, Long JC (1992) Matrix correlation analysis in anthropology and genetics. Yearbook Physiological Anthropology, 35, 187-213.

Smouse PE, Long JC, Sokal RR (1986) Multiple regression and correlation extensions of the Mantel test of matrix correspondence. Systematic Zoology, 35, 627-632.

Stoate C, Báldi A, Beja P, Boatman ND, Herzon I, van Doorn A, de Snoo GR, Rakosy L, Ramwell C (2009) Ecological impacts of early 21st century agricultural change in Europe – A review. Journal of Environmental Management, 91, 22-46.

Temple HJ, Hoffman JI, Amos W (2006) Dispersal, philopatry and intergroup relatedness: fine-scale genetic structure in the white-breasted thrasher, *Ramphocinclus brachyurus*. Molecular Ecology, 15, 3449-3458.

Teunissen W, Soldaat L (2005) Indexen en trends van een aantal weidevogelsoorten uit het Weidevogelmeetnet. Periode 1990-2004. SOVON-informatie 2005/13 SOVON Vogelonderzoek Nederland Beek-Ubbergen.

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.

van den Brink V, Schroeder J, Both C, Lourenco PM, Hooijmeijer JCEW, Piersma T (2008) Space use by Black-tailed Godwits *Limosa limosa limosa* during settlement at a previous or a new nest location. Bird Study, 55, 188-193.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes, 4, 535–538.

van Treuren R, Bijlsma R, Tinbergen JM, Heg D, van de Zande L (1999) Genetic analysis of the population structure of socially organized Oystercatchers (*Haemotoopus ostralegus*) using microsatellites. Molecular Ecology, 8, 181-187.

van Turnhout CAM, Foppen RPB, Leuven RSEW, Siepel H, Esselink H (2007) Scale-dependent homogenization: Changes in breeding bird diversity in the Netherlands over a 25-year period. Biological Conservation, 134, 505-516.

Verkuil YI, Trimbos K, Haddrath O, Baker AJ (2009) Characterization of polymorphic microsatellite DNA markers in the black-tailed godwit (*Limosa limosa: Aves*). Molecular Ecology Resoures, 9, 1415-1418.

Vucetich JA, Waite TA (2001) Migration and inbreeding: the importance of recipient population size for genetic management. Conservation Genetics, 2, 167-171.

Wolf JBW, Bayer T, Haubold B, Schilhabel M, Rosenstiel P, Tautz D (2010) Nucleotide divergence vs. gene expression differentiation: comparative transcriptome sequencing in natural isolates from the carrion crow and its hybrid zone with the hooded crow. Molecular Ecology, 19, 162-175.

Woxvold IA, Adcock GJ, Mulder RA (2006) Fine-scale genetic structure and dispersal in cooperatively breeding apostlebirds. Molecular Ecology, 15, 3139-3146.

Zink RM, Barrowclough GF (2008) Mitochondrial DNA under siege in avian phylogeography. Molecular Ecology, 17, 2107-2121.

Zwarts L, Bijlsma RG, van der Kamp J, Wymenga E (2009) Living on the edge. Wetlands and birds in a changing Sahel. KNNV Publishing Zeist.

CHAPTER

5

Genetic differentiation between Dutch Black-tailed Godwit breeding sites re-examined using D statistics

Wader Study Group Bulletin (2012), 119, 59-60

K.B. Trimbos and C.J.M. Musters

Abstract

In previous research genetic structure between different breeding areas in The Netherlands was investigated. Between 9 different breeding areas no genetic differentiation was found calculating F_{ST} values only. However these calculations were based on highly polymorphic microsatellite data, which could pose the problems for traditional, $F_{ST,}$ genetic differentiation calculations. Therefore we re-analyzed our previous dataset using Jost's D statistics to assess genetic differentiation. The re-assessment of genetic differentiation between Dutch Black-tailed Godwit breeding areas, using D statistics, indicated that these areas are not genetically different from each other. Like in the previous study these data yet again indicate that the Dutch Black-tailed Godwit breeding areas should be considered and managed as one panmictic unit.

Keywords Molecular ecology · Genetic population structure · F statistics · Limosa limosa · Breeding population · Isolation · Habitat fragmentation

Introduction

The assessment of population genetic structure has proven to be a powerful descriptive tool in the field of conservation genetics, molecular ecology, evolutionary studies and forensics. The method most frequently used to assess genetic population structure to date is F statistics, introduced by Sewall Wright (1943; 1965). However, recent reports point at the limitations of F-statistics for genetic differentiation calculations in studies using highly variable loci (Gregorius *et al.* 2007, Gregorius 2010, Jost 2008). Jost (2008) points out that the dependency of these statistics on within-population diversity gives unreliable results using these statistics when diversity and differentiation are high. Using F-statistics, similarity between subpopulations (F_{ST}) is measured as the ratio of within-population heterozygosity (H_S) to total heterozygosity (H_T). F_{ST} was originally developed for bi-allelic markers, with F_{ST} values close to zero supposedly indicating high similarity among populations, while values close to unity supposedly indicate nearly complete differentiation (Wright 1943; 1965). However, with multi-allellic markers the maximum possible value equals to 1- H_S (Hedrick 2005). Highly variable loci result in a high H_S value that will reduce the possible range of F_{ST} considerably. For example when within-population heterozygosity is 0.9, which is a common value when using microsatellite markers, the maximum possible value of F_{ST} = 0.1, which is generally interpreted as representing weak population subdivision. However, in this example it represents the case with no shared alleles among populations, and maximum divergence (Meirmans and Hedrick 2011). Jost (2008) indicates that this dependency of F_{ST} on the level of within population diversity will cause difficulties in its interpretation, for example when comparing F_{ST} values of groups with different genetic diversity, markers exhibiting different mutation rates, or species with different effective population sizes. Another problem indicated by Jost (2008) is that the expected heterozygosity alone is unsuitable for describing the genetic diversity. Jost (2008) developed a new statistic for estimating differentiation which he termed 'D', avoiding these problems by using the effective number of alleles instead of heterozygosity. Jost (2008) shows that assessing datasets with D that were formerly assessed with F_{ST} might give very different results.

In response to agricultural intensification, which began its rise around 1900 in western Europe, the Black-tailed Godwit, increased in population numbers. However, continuing agricultural intensification has resulted in decreasing population numbers in most of the Black-tailed Godwit's European breeding range (Beintema *et al.* 1995, Bijlsma *et al.* 2001). From 1967 an annual and steady decline in population numbers has also been reported in The Netherlands, one of the remaining Black-tailed Godwit strongholds in Europe (Teunissen and Soldaat 2005). The Black-tailed Godwit shows high breeding site fidelity and some degree of natal philopatry. It was shown that 90% of the adult breeding birds returned within 700 m of the previous nest site. Natal philopatry was demonstrated to be high as well, with 75% of the birds returning within 18 km of their previous hatching site (Groen 1993). With such limited dispersal in a fragmenting landscape, breeding areas could become isolated from each other, resulting in isolated populations. These isolated populations might have small effective population sizes which might affect genetic diversity through genetic drift and might increase inbreeding. Isolation might also affect population dynamics, resulting in a metapopulation structure including source-sinks dynamics. To get some more insight into the population dynamics of

Black-tailed Godwit breeding populations, we assessed genetic structure between different breeding areas in The Netherlands in a former study (Trimbos *et al.* 2011). Between 9 different breeding areas no genetic differentiation was found calculating F_{ST} values only. However these calculations were based on highly polymorphic microsatellite data, which could pose the problems pointed out by Jost (2008). Therefore we re-analyzed our previous dataset using Jost's D (2008) to assess genetic differentiation. Results of both statistics will be compared and discussed here.

Material and methods

Samples, sample locations (Zuid-West Fryslân (ZWF), Eemnes/Arkemheen/Vinkeveen (EAV), Zoeterwoude (ZOE), Idzegea (IDZ), Middelie (MID), Normerpolder (NOR), Vijfheerenlanden (VIJF), Uitdam (UIT) and OVE (Overijssel) and the according PCR fragment dataset from Trimbos *et al.* (2011) was used for genetic differentiation calculations. Pairwise F_{ST} values between locations were previously calculated using ARLEQUIN 3.11 and are used here again (Excoffier *et al.* 2005). If significant values were obtained, Bonferroni correction was applied. D values between different locations were calculated using the Species Prediction And Diversity Estimation (SPADE) program with 2000 bootstrap replications (Chao and Shen 2009). Confidence intervals (CI), calculated by the program, were used to assess if D values differed from zero.

Results

Nearly all pairwise F_{ST} values between locations were not significantly different from zero, except ZWF and VIJF (Table 1). However, after Bonferroni correction this significance did not hold. Most D values were 0. Three D values, between UIT and NOR, ZWF and VIJF and between EAV and VIJF were apparently higher than 0. However, their confidence intervals overlapped with 0.

Table 1. Pairwise F_{ST} below the diagonal, and D values above the diagonal for pairs of Dutch Black-tailed godwits populations. Uncorrected P values smaller than 0.05, for F_{ST}, or CI's not overlapping with zero, for D values, are indicated with *.

	ZWF	EAV	ZOE	IDZ	MID	NOR	VIJF	UIT	OVE
ZWF	--	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000
EAV	0.005	--	0.000	0.000	0.000	0.000	0.003	0.000	0.000
ZOE	0.000	0.000	--	0.000	0.000	0.000	0.000	0.000	0.000
IDZ	0.000	0.004	0.000	--	0.000	0.000	0.000	0.000	0.000
MID	0.000	0.001	0.000	0.000	--	0.000	0.000	0.000	0.000
NOR	0.002	0.000	0.000	0.003	0.000	--	0.000	0.016	0.000
VIJF	0.013*	0.015	0.008	0.003	0.000	0.000	--	0.000	0.000
UIT	0.000	0.000	0.000	0.000	0.000	0.013	0.000	--	0.000
OVE	0.003	0.006	0.000	0.001	0.000	0.000	0.005	0.000	--

Discussion

The re-assessment of genetic differentiation between Dutch Black-tailed Godwit breeding areas, using D statistics, indicates that these areas are not genetically different from each other. Most D values were 0 or close to 0 indicating no differentiation. The D value of 0.016, between UIT and NOR, might indicate very slight genetic differentiation between these sites. However, the CI indicated that this value was not different from 0. In general these results seem comparable to the earlier calculated pairwise F_{ST} values between Dutch Black-tailed Godwit breeding sites. Pairwise F_{ST} values were low and only one pairwise comparison, between VIJF and ZWF, was significantly different from 0, indicating that these 2 locations might be genetically differentiated from each other. However this significant F_{ST} value was lost after Bonferroni correction.

Does this mean that F_{ST} and D performed equally and that both statistics indicate that there is no genetic differentiation? Jost (2008) seems to argue that only D should be used to assess genetic differentiation. Meirmans and Hedrick (2011) point out that while D performs better at measuring the actual differentiation between demes, F_{ST} is better at describing the influence of demographic events on the distribution of alleles and suggest to use both statistics simultaneously to get a better comprehension of population structure. In this view, D values indicates an absence of genetic divergence while F_{ST} values indicate a lack of allele fixation between breeding areas. This more complete assessment of genetic structure demonstrates that the Dutch Black-tailed Godwit breeding areas are still genetically similar or have become isolated from each other too recently for lineage sorting to become complete. These data yet again do not indicate that the Dutch Black-tailed Godwit breeding areas should not be considered and managed as one panmictic unit.

References

Beintema A, Moedt O, Ellinger D (1995) Ecologische Atlas van de Nederlandse Weidevogels. Schuyt and Co, Haarlem.

Bijlsma RG, Hustings F, Camphuysen CJ (2001) Algemene en schaarse vogels van Nederland (Avifauna van Nederland 2). Haarlem/Utrecht: GMB Uitgeverij/KNNV Uitgeverij.

Chao A and Shen T-J (2009) *Program SPADE (Species Prediction and Diverstiy Estimation)*. Program and user's guide a http://chao.stat.nthu.edu.tw.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, 1, 47-50.

Gregorius HR, Degen B, Koenig A (2007) Problems in the analysis of genetic differentiation among populations – a case study in *Quercus robur*. Silvae Genetica, 56, 190-199,

Gregorius HR (2010) Linking diversity and differentiation. Diversity, 2, 370-394.

Groen NM (1993) Breeding site tenacity and natal philopatry in the Black-tailed Godwit. Ardea, 81, 107-113.

Jost L (2008) G_{ST} and its relatives do not measure differentiation. Molecular Ecology, 17, 4015-4026.

Hedrick PW (2005) A Standardized Genetic Differentiation Measure. Evolution, 59, 1633-1638.

Meirmans PG and Hedrick PW (2011) Assessing population structure: F_{ST} and related measure. Molecular Ecology Resources, 11, 5-8.

Rice WR (1989) Analysing tables of statistical tests. Evolution, 43, 223-225

Teunissen W, Soldaat L (2005) Indexen en trends van een aantal weidevogelsoorten uit het Weidevogelmeetnet. Periode 1990-2004. SOVON-informatie 2005/13 SOVON Vogelonderzoek Nederland Beek-Ubbergen.

Trimbos KB, Musters CJM, Verkuil Y, Kentie R, Piersma T and de Snoo GR (2011) No evident spatial genetic structuring in the rapidly declining Black-tailed Godwit *Limosa limosa limosa* in The Netherlands. Conservation Genetics, 12, 629-636.

Wright S (1943) Isolation by distance. Genetics, 28, 114.

Wright S (1965) The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution, 19, 395-420.

CHAPTER

6

Patterns in nuclear and mitochondrial DNA reveal historical and recent isolation in the Black-tailed Godwit *(Limosa limosa)*

PLOS One (Submitted)

Krijn B. Trimbos, Camiel Doorenweerd, Ken Kraaijeveld, C.J.M. Musters, Niko M. Groen, Peter de Knijff, Theunis Piersma and Geert R. de Snoo

Abstract

On the basis of morphological differences, three subspecies of Black-tailed Godwit (*Limosa limosa*) have been recognized (*L. l. limosa, L. l. islandica* and *L. l. melanuroides*). In previous studies mtDNA sequence data showed minimal genetic divergence between the three subspecies and an absence of substructuring within *L. l. limosa*. Here, population genetic structure and phylogeographic patterns have been analyzed using COI, HVR1 and HVR2 mtDNA sequence data as well as 12 microsatellite loci (nuDNA). The nuDNA data suggest genetic differentiation between *L. l. limosa* from Sweden and The Netherlands, between *L. l. limosa* and *L. l. islandica*, but not between *L. l. limosa* and *L. l. melanuroides*. However, the mtDNA data were not consistent with the nuDNA pattern. mtDNA did support a split between *L. l. melanuroides* and *L. l. limosa*/*L. l. islandica* and also demonstrated two *L. l. limosa* haplotype clusters that were not geographically isolated. This genetic structure can be explained by a scenario of isolation of *L. l. melanuroides* from *L. l. limosa* in Beringia during the Last Glacial Maximum, possibly followed by recent introgression. During the Pleistocene separation of *L. l. islandica* from *L. l. limosa* occurred, followed by colonization of Iceland by the *L. l. islandica* during the Holocene. Within *L. l. limosa* founder events, followed by population expansion, took place during the Holocene also. According to the patterns observed in both markers together and their geographic separation, we propose that the three traditional subspecies indeed represent three separate genetic units.

Keywords DNA barcode · Incomplete lineage sorting · Microsatellites · Population Genetics · Pleistocene · Subspecies

Introduction

Until a few centuries ago, breeding Black-tailed Godwits (*Limosa limosa* Linneaus, 1785) were confined to raised bogs, moorlands, lake margins and damp grassy depressions in steppe (Beintema *et al.* 1995, Haverschmidt 1963). Since the early Middle Ages the bog habitats in north-western Europe became converted into increasingly nutrient-rich meadows for dairy farming. Black-tailed Godwits were probably quick to exploit this new opportunity and as a result the number of breeding pairs in The Netherlands alone increased to approximately 120,000 in 1967 (Mulder 1972). However, over the last few decades further agricultural intensification with ever earlier mowing dates has led to low recruitment (Kleijn *et al*. 2010, Schekkerman *et al.* 2008). In addition, urbanization of rural areas has led to fragmentation of their breeding habitat. As a result, the mainland European breeding population has been in decline over the last 40 years (Bijlsma *et al.* 2001, Birdlife international 2004, Zwarts *et al.* 2010, Schekkerman *et al.* 2008). This has prompted the IUCN to qualify the species as Near-Threatened (www.birdlife.org/datazone/speciesfactsheet.php?id=3003).

Black-tailed Godwits are migratory shorebirds breeding mainly in temperate and boreal lowlands. Their breeding range extends across Eurasia, from Iceland to Kamchatka and Sakhalin (Cramp and Simmons 1982). Currently, three subspecies are recognized within this range (Figure 1): the European Black-tailed Godwit (*L. l. limosa*), Icelandic Black-tailed Godwit (*L. l. islandica*) and Asian Black-tailed Godwit (*L. l. melanuroides*) (Cramp and Simmons 1982). These subspecies have been distinguished on the basis of morphological traits. *L. l. islandica* has a shorter bill and tarsus and has more extensive rufous-cinnamon and barred plumage than *L. l. limosa*, while *L. l. melanuroides* is distinctly smaller compared with *L. l. limosa* (Cramp and Simmons 1982, Roselaar and Gerritsen 1991). Aside from morphological differences, the subspecies also differ in breeding range and migratory routes, although there is some overlap (Gill *et al.* 2007). The breeding range of nominate *L. l. limosa* extends from Britain to West Russia. *L. l. islandica* breeds mainly on Iceland, with some breeding pairs occasionally found in Scotland and Northern Norway. *L. l. melanuroides* breeds at isolated locations in Russia east of the Yenisey river. *L. l. limosa* winters in parts of southern Europe and south-west Asia, but mainly in sub-Saharan Africa. *L. l. islandica* migrates to Britain, western France, The Netherlands and Iberia. The wintering grounds of *L. l. melanuroides* are in south-east Asia, from the Bay of Bengal to Taiwan, the Philippines and Australia (Cramp and Simmons 1982, Gill *et al.* 2007).

Höglund *et al.* (2009) found slight diagnostic differences between the subspecies on the basis of mitochondrial DNA (mtDNA) sequence data, but found no population structure within *L. l. limosa*. Although they had sequenced part of the highly variable control region (CR) of the mtDNA, they used a relatively conserved part in their analyses (Liebers *et al.* 2001, Ruokonen and Kvist 2002). Using microsatellite markers (nuDNA), Trimbos *et al.* (2011) found moderate levels of genetic variation among Black-tailed Godwits breeding in The Netherlands, and also failed to detect any form of population structure. This suggests either that fragmentation of Black-tailed Godwit breeding populations is too recent for lineage sorting to be complete, or that gene flow has not been restricted. However, genetic structure has yet to be studied throughout the entire breeding range of the Black-tailed Godwit.

Owing to its four times smaller effective population size, mtDNA exhibits faster lineage sorting compared with nuDNA (Moore 1998, Rubinoff *et al.* 2006, Zink and Barrowclough 2008). This difference in effective population size is attributed to the different ways in which the two genomes are inherited. Nuclear DNA is diploid, and recombined between both parents in every generation, whereas mtDNA is haploid and only inherited maternally. In theory, then, mtDNA could thus reflect changes in population structure faster. It has been argued, however, that the best measures of population genetic structure derive from the accumulated signals from multiple loci (Edwards and Bensch 2009), while the entire mtDNA is effectively a single locus. With this in mind, we used a combination of both nuDNA and mtDNA data to account for the shortcomings of each DNA type (Rubinoff and Holland 2005, Mantooth and Riddle 2011). More specifically, we first determined the DNA barcode, part of the mitochondrial Cytochrome C Oxidase I (COI) gene, for a subset of samples. Today, there is a large and growing database of COI barcodes (Barcoding of Life datasystems, www.boldsystems.org), including barcodes for many bird species (Schindel *et al.* 2011). COI data allowed for easy comparison of the results from our samples with those of other studies. Secondly, we used next-generation sequencing on the Illumina HiSeq platform to determine primer sites for the amplification of the hypervariable regions HVR1 and HVR2 of the mitochondrial control region (CR). For the nuDNA data we used a set of 12 microsatellite markers (Verkuil *et al.* 2009)used previously in Trimbos *et al.* 2011.

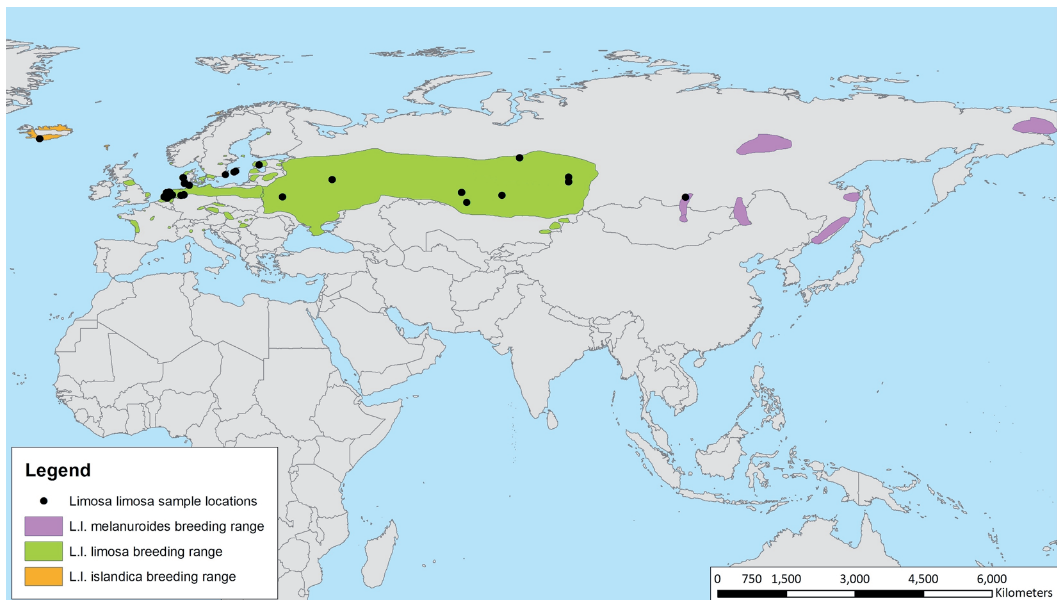


Figure 1. Sample locations of the Black-tailed Godwit *Limosa limosa*. *L. l. limosa*: the Netherlands, Mid-Germany, Northern Germany, Denmark, Sweden, Belarus/Moscow, Kazachstan/SW Russia; *L. l. islandica*: Iceland; and *L. l. melanuroides*: Eastern Russia/Selanga delta.

Material and methods

Sample collection and DNA extraction

Samples were collected between 1991 and 2010 from sites across the *Limosa limosa* breeding range (Figure 1). In The Netherlands, blood samples were collected as described in Trimbos *et al.* (2011). Other blood samples, previously collected (in Sweden, Russia/Moscow, Kazakhstan, western Russia, Iceland, Eastern Russia/Selenga Delta and Canada) by Höglund *et al.* (2009), were made available by the University of Groningen, where they were stored (Table 1). Blood was stored in 97% alcohol at -70 °C.

Table 1. Sample location, exact geographical location, number of samples per geographical location used for microsatellite analysis (nuDNA), number of samples per sample location used for microsatellite analysis (msat pooled), number of samples per geographical location used for mtDNA analysis (mtDNA), number of samples per sample location used for mtDNA analysis (mtDNA pooled) and the *Limosa* species or *Limosa limosa* subspecies per sample location.

Sample location	Exact geographical location	nuDNA	nuDNA pooled	mtDNA	mtDNA pooled	Limosa species/ subspecies
Netherlands (140)	Eemnespolder/Arkemheen	24	140	6	46	*Limosa limosa limosa*
	Grote Zoeterwoudse polder	11		4		*Limosa limosa limosa*
	Vijfheerenlanden	10		4		*Limosa limosa limosa*
	Uitdam	11		4		*Limosa limosa limosa*
	Polder Zeevang	11		4		*Limosa limosa limosa*
	Normerpolder	7		4		*Limosa limosa limosa*
	Overijssel/Zwolle	10		4		*Limosa limosa limosa*
	South-west Frysland	38		12		*Limosa limosa limosa*
	Idzegea	18		4		*Limosa limosa limosa*
Germany (35)	Mid-Germany, Schneckenbruch	3	35	3	13	*Limosa limosa limosa*
	Mid-Germany, Dummer	20		6		*Limosa limosa limosa*
	Northern Germany, Fohr	11		3		*Limosa limosa limosa*
	Northern Germany, Meggerdorf	1		1		*Limosa limosa limosa*
Denmark (11)	Tipperne	11	11	4	4	*Limosa limosa limosa*
Belarus (6)	Belarus	3	6	3	6	*Limosa limosa limosa*
	Moscow	3		3		*Limosa limosa limosa*
Sweden (42)	Kristianstad/Faludden/ Hummelbosholm/Oland	42	42	4	4	*Limosa limosa limosa*
Kazakhstan, SW Russia (23)	Novosibirsk	5	23	2	8	*Limosa limosa limosa*
	Lake Ubinsky	2		0		*Limosa limosa limosa*
	Lake Sharkol	5		2		*Limosa limosa limosa*
	Lake Baituma	2		1		*Limosa limosa limosa*
	Lake Big Aksuhat	1		1		*Limosa limosa limosa*
	Lake Shoskaly	2		1		*Limosa limosa limosa*
	Juganski	6		1		*Limosa limosa limosa*
Iceland (27)	W. Iceland	27	27	5	5	*Limosa limosa islandica*
Eastern Russia (3)	River Selenga Delta	3	3	3	3	*Limosa melanuroides*
Canada (2)	Churchill, Manitoba	2	2	2	2	*Limosa heamastica*

Additionally, eggshells were obtained between 2008 and 2010 (Trimbos *et al.* 2009) in The Netherlands, Germany, Belarus and Denmark, all breeding areas of *L. l. limosa* (Table 1). Eggshell remains were collected in the nest (after hatching) and were individually stored in plastic bags at room temperature. DNA was extracted from 6-10 µl of blood using ammonium acetate (Richardson *et al.* 2001) or the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's protocol (Qiagen 2003). DNA from eggshell membranes was also extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen 2003), with minor modifications as described by Trimbos *et al.* (2009). Publicly available sequences from the Barcoding of Life Database (BOLD) were used to supplement the COI barcodes and to provide an outgroup for the COI tree. The Hudsonian Godwit *Limosa haemastica*, an arctic-breeding godwit of Canada and Alaska, was used as outgroup for the HVR analysis.

Microsatellite analysis

A total of 289 birds from 10 different breeding locations were genotyped at 12 microsatellite loci. These 12 loci (LIM3, LIM5, LIM8, LIM10, LIM11, LIM12a, LIM24, LIM25, LIM26, LIM30, LIM33) were specifically developed for Black-tailed Godwits (Verkuil *et al.* 2009). A Fisher's exact test for linkage disequilibrium was carried out using all 289 samples, with 1,000 dememorization steps, 100 batches and 1,000 iterations per batch (GENEPOP web version 4.0; Raymond and Rousset 1995). Deviations from Hardy-Weinberg equilibrium and heterozygote excess or deficiency were tested for each locus and sampling location separately using 1,000 dememorization steps, 100 batches and 1,000 iterations per batch (GENEPOP; Raymond and Rousset 1995). Bonferroni correction for multiple testing was applied (Rice 1989). To detect scoring and amplification errors, we employed MICRO-CHECKER with a 95% confidence interval over 10,000 runs (Oosterhout *et al.* 2004).

For each location, observed (H_o) and expected (H_e) heterozygosities and inbreeding values (F_{IS}) were estimated using ARLEQUIN 3.11 (Excoffier *et al.* 2005) set at 20,000 permutations. An analyses of molecular variance (AMOVA) was performed, allowing variance among sample locations (V_a), variance within sample locations (V_b) and residual variance to be computed (V_c), using ARLEQUIN with 20,000 permutations, followed by Bonferroni correction. Additionally, D was calculated using SPADE (Cho *et al.* 2008), as recent studies have shown that this statistic provides more accurate estimates of genetic differentiation than Fst (Jost 2008; Meirmans and Hedrick 2011). The number of private alleles was determined using CONVERT 1.31 (Glaubitz 2004). FSTAT 2.9.3.2 (Goudet 1995) was used to calculate allelic range, number of alleles per sample location and allelic richness per sample location. To correct for sample size, this program uses the rarefaction index, as described by Hurlbert (1971).

Historic and recent gene flow were investigated using MIGRATE v 3.2.7 (Beerli and Felsenstein 1999;2001) and BAYESASS (Wilson and Rannala 2003). MIGRATE uses coalescent theory in combination with maximum likelihood or Bayesian calculations and MCMC sampling to estimate the relative effective population size (4Neµ) and asymmetric gene flow *M* (m/µ) over approximately 4Ne generations in the past (Beerli 2008). We ran MIGRATE three times using the Bayesian inference; run 1 sampled 200,000 values (1 long chain, 5000 recorded steps, an increment of 10 and 4

replicate chains), run 2 sampled 400,000 (1 long chain, 5000 recorded steps, an increment of 10 and 8 replicate chains) and run 3 sampled 800,000 values (1 long chain, 8000 recorded steps, an increment of 10 and 10 replicate chains). Four-chain heating at temperatures of 1, 1.5, 3 and 10000 was implemented to increase the efficiency of the MCMC. 4Neμ and *M* were compared between the three runs, as were the Bayesian posterior distribution graphs to ascertain whether chains had converged. Here, the results from the longest run are presented, as posterior distribution graphs showed convergence for most of the pairwise comparisons and 4Neμ and *M* values were similar between runs. BAYESASS uses a Bayesian approach and MCMC sampling to generate *m* values which reflect gene flow over the last 5 generations (Wilson and Rannala 2003). BAYESASS was run as described by Chiucchi and Gibbs (2010), with one small modification. The gene flow results of the short run that fit the data best according to a Bayesian deviance measure (Chiucchi and Gibbs 2010) were not comparable with the results of the longer run with the same parameter settings, indicating that with these settings the runs did not converge. We therefore chose the run that fit the data second best, as this run did show similar results in longer runs with the same parameter settings. It is these data that are presented here.

STRUCTURE 2.3.1 (Pritchard *et al.* 2000) was used to cluster genotypes from all sampling locations. We determined the deltaK (Structure Harvester), a calculation of the second-order rate of change in log likelihood Ln P(X|K), as recommended by Evanno *et al.* (2005). The most likely number of genetic clusters (K) in our sample set was also investigated by determining the maximum average log likelihood Ln P(X|K). Values computed with both methods were plotted using Structure Harvester 0.56.3 (Dent 2009, web version). The Structure model was run using admixture and correlated allele frequencies. Additionally, the LOCPRIOR model, incorporated into STRUCTURE 2.3.1, was used. This model assumes that individuals sampled close together are often from the same population and can assist in the clustering when population structure is weak. The program was run 5 times with a burn-in period of 200,000 iterations and a length of 1,000,000 MCMC iterations for K (1-13). Convergence was assessed by checking whether the graphs provided by the program reached equilibrium before the end of the burn-in phase. CLUMPP was used to estimate the number of identical repeat runs per K. The output of CLUMPP was accordingly used to generate graphs from the STRUCTURE results using Microsoft Excel.

A Mantel test with 9999 permutations was performed using GENALEX 6.2 (Peakall and Smouse 2006) to test for correlation between the genetic and geographic distance matrices (Smouse and Long 1992, Smouse *et al.* 1986).

Mitochondrial DNA sequencing

To identify suitable primer sites around the hypervariable sites (HVR1 and HVR2) in the control region of the mtDNA, we sequenced the entire mtDNA of three *L. l. limosa* samples (from The Netherlands, Sweden and SW Russia) at low coverage. For each sample, 1000 ng of genomic DNA was sheared to 500bp fragments using a Covaris S2. These fragments were end-repaired and fitted with an A-overhang at the 3' end using NEBNext TruSeq. Adapters were ligated to these

fragments, after which they were sequenced on an Illumina HiSeq2000. The resulting reads were aligned against the complete mitochondrial sequence of the Ruddy Turnstone *Arenaria interpres* (Paton *et al.* 2002) using Stampy (http://www.well.ox.ac.uk/project-stampy). Barcoded DNA pools sequenced on part of a single lane of an Illumina HiSeq resulted in 817,335, 6,804,981 and 3,273,078 paired-end reads from *L. limosa* samples from the Netherlands, Sweden and SW Russia, respectively. Alignment of the Illumina reads to the *A,interpres* mtDNA with the substitution rate set to 0.1 resulted in 982, 10,068 and 806 aligned reads, respectively. These covered the mtDNA genome 0.58, 9.37 and 2.6 times, respectively. A consensus sequence was constructed using Samtools pileup (http://samtools.sourceforge.net/). On the basis of this consensus sequence, primers were developed amplifying the first and third domain of the *L. limosa* CR (5'-3'; F-primer: L13F – AGCAGTTCCTGCTTGGCTTT, R-primer: L13R – GCAAGTTGTGCTAGGGGTTT and 5'-3'; F-primer: L23F – TTCAAGTGTCCGGGGAATCA, R-primer: L23R TTTGTCTCTGGGTGCATGGG). As sequencing with L13F and L23R proved to be problematic owing to long T-trains and CAAACAAAA repeats, further sequencing was performed unidirectionally using only primers L13R and L23F. For HVR1 and HVR2, 649 bp were sequenced in 91 samples, including 81 *L. limosa* individuals from 23 different *L. l. limosa* breeding locations, five *L. l. islandica* individuals from Iceland, three individuals *L. l. melanuroides* from Eastern Russia and two *L. haemastica* individuals (Table 1). However, for other HVR1 and HVR2 analysis five sequences of poor quality, including the two samples from *L. haemastica* were excluded, adding up to a sample set of 78 samples from *L. l. limosa* breeding locations, five *L. l. islandica* from Iceland and three *L. l. melanuroides* from Eastern Russia.

The universal COI mitochondrial barcode region was amplified using primers BirdF1, BirdR1 and BirdR2 with the addition of M13 tails (Hebert *et al.* 2004). A cocktail of all three primers was used to increase PCR success rate. A section of 658 bp of the COI gene was sequenced for a subset of 56 samples, which included 52 individuals from several *L. l. limosa* breeding locations, three *L. l. islandica* from Iceland and one *L. l. melanuroides* from Eastern Russia.

PCR amplification reactions for L13 and L23 primer pairs were carried out in a total volume of 25µl consisting of 10 ng genomic DNA, 2.5µl PCR Buffer 10x including 15 mM $MgCl_2$, 2.5mM dNTP, 110 pmol of each primer, 1.25 U Taq DNA polymerase (Qiagen) and 18.8 µl DNA mQ water. For COI the same volume and PCR mix reagents were used with the exception of the amount of primer, which was now 250 pmol of each primer (M13F-BirdF1, M13R-BirdR1 and M13R-BirdR2). PCR was conducted on a BIORAD S1000 thermal cycler using the following PCR program: 94°C for 3 min; 40 cycles of 94°C for 15 s, locus-specific Ta 30 s, 72°C 40 s; 72°C for 5 min. Ta was 50°C for COI and 58°C for L13 and L23. With each PCR a negative control was included and sequenced to check for contamination issues. Sequencing was outsourced to Macrogen Europe. Forward and Reverse chromatograms were combined in Sequencer v4.10.1 (Gene Codes Coorporation), checked manually for ambiguities, exported as FASTA files and aligned using BioEdit v7.0.9 (Hall 1999). All novel sequences generated for this study are deposited at GenBank (accession numbers JQ657268-JQ657500).

Mitochondrial DNA analysis

For HVR1 and HVR2 the number of haplotypes, haplotype diversity and nucleotide diversity were calculated using dnaSP v5.0 (Librado and Rozas 2009), with gaps excluded as potential sequence variability. To detect past population expansions we calculated Fu's F_S statistic and Tajima's D-test (Fu 1997, Tajima 1989). To test for background selection Fu and Li's D* and F* statistics were used (Fu and Li 1993). To obtain pairwise Φst between sampling sites, pairwise Juke and Cantor distances and haplotype frequencies were calculated in ARLEQUIN 3.11 (Excoffier *et al.* 2005) with 20,000 permutations. A median-joining haplotype network was constructed using NETWORK v. 4600 (Fluxus-engineering).

DNA barcodes are available for 91% of all bird species (Schindel *et al.* 2011), allowing for a comparison of the genetic variation of the mtDNA within *Limosa limosa* with other bird species (Kerr *et al.* 2007, Schindel *et al.* 2011). As DNA barcoding aims to identify species, the BOLD data structure does not recognize subspecies. However, subspecies clusters were recognized nonetheless through our own added subspecies COI sequence data and comments in the 'notes' field in some BOLD records. Phylogenetic analysis of HVR1 and HVR2 was performed using two different approaches, Bayesian and maximum likelihood, using *L. haemastica* (CAN) as outgroup. For this analysis, samples with missing sequence data were included with this data part encoded as 'missing data'. The Akaike Information Criterium in MrModeltest v2.3 (Nylander 2004) concluded that the HKY + G model was most suitable for the combined HVR1 and 2 dataset. MrBayes (Huelsenback and Ronquist 2001) was subsequently used for the Bayesian analysis, with the HKY + G model, a melting temperature of 0.01, two runs of four chains each, a burn-in of 1 million and a total of 10 million generations. PhyML (Guindon and Gascuel 2003) was used for the maximum likelihood analysis, with the HKY85 substitution model and 10,000 bootstrap generations. For COI a neighbor-joining tree was created using PAUP* 4.10b with uncorrected P distance (Srivathsan and Meijer 2011).

Results

Microsatellite analysis (nuDNA)

A total of 132 different alleles were amplified. The number of alleles per locus ranged from 4 to 15, with no more than 2 alleles per individual. After sequential Bonferroni correction the breeding populations in The Netherlands showed a significant global heterozygote deficit at 6 loci, indicating low heterozygosity in this population. No significant linkage disequilibrium was found between any of the loci after sequential Bonferroni correction. MICROCHECKER detected no null alleles at any of the loci in the complete dataset.

For each sampling location, Table 2 reports the absolute number of alleles, allelic richness, F_{IS}, and private alleles. Neither *L. l. islandica* nor *L. l. melanuroides* showed the presence of private alleles.

Table 2. Sample location and *Limosa limosa* subspecies; number of HVR1 and HVR2 sequence alignments (n), nucleotide diversity (pi), haplotype diversity (h), number of haplotypes (nh) for mtDNA; and number of individuals (n), absolute number of alleles (A), allelic richness (A_R), number of private alleles (P_a) and inbreeding coefficient (F_{IS}) for microsatellite fragment analysis (Msats).

Sample location / *L. limosa* subspecies	mtDNA (n)	pi	h	nh	Msats (n)	A	A_R	P_a	F_{IS}
Netherlands *Limosa limosa limosa*	46	0.006	0.896	16	140	123	2.689	11	0.041*
Mid-Germany *Limosa limosa limosa*	9	0.007	0.972	8	23	84	2.673	0	-0.023
Northern Germany *Limosa limosa limosa*	4	0.007	1.000	4	12	70	2.582	0	0.072
Denmark *Limosa limosa limosa*	4	0.009	0.833	3	11	68	2.579	1	0.002
Belarus/Moscow *Limosa limosa limosa*	6	0.009	1.000	4	6	55	2.581	0	0.189*
Sweden *Limosa limosa limosa*	4	0.002	0.500	2	42	100	2.656	3	0.034
Kazachstan/SW Russia *Limosa limosa limosa*	8	0.005	0.929	6	23	97	2.695	4	-0.002
Iceland *Limosa limosa islandica*	5	0.008	0.900	4	27	62	2.355	0	0.054
Eastern Russia *Limosa limosa melanuroides*	3	0.010	0.667	2	3	41	2.667	0	0.143

F_{IS} values were significantly different from zero in The Netherlands and Belarus. AMOVA calculations showed significance for all the calculated variances (3% Va = 0.03 P < 0.0001, 3% Vb = 0.04 P = 0.0001 and 94% Vc = 0.06 P < 0.0001). The molecular variance present in the sample set was explained for 3% by differences between sample locations. An additional 3% of the variance was explained by differences between individuals within locations. The remaining 94% was randomly distributed over populations, indicating the existence of genetic differentiation, although small, between populations. D supported differentiation between samples from Iceland and the other sampling locations (Table 3). Also, D indicated weak but significant differentiation between Dutch and Swedish samples (Table 3).

Table 3. Below the diagonal: D values for the microsatellite loci; above the diagonal: pairwise Φst for mtDNA HVR1 and HVR2 sequences. CIs not overlapping with zero for D values and significant P values after sequential bonferroni correction for Φst are indicated by *.

	Netherlands	M Germany	N Germany	Denmark	Belarus	Sweden	Kaz/W Rus	Iceland	E Russia
Netherlands	-	-0.03529	-0.06135	-0.04698	-0.05796	0.23901	-0.00394	0.53332*	0.91115*
M Germany	0.005	-	-0.13251	-0.11034	-0.09773	0.32468	-0.05243	0.51159	0.91407
N Germany	0.026	0.022	-	-0.21049	-0.11098	0.33619	-0.13143	0.47302	0.92038
Denmark	0.009	0.029	0.018	-	-0.06555	0.30287	-0.09829	0.44205	0.90956
Belarus	0.000	0.039	0.037	0.030	-	0.39894	-0.09067	0.51269	0.92515
Sweden	0.022*	-0.010	0.036	0.027	0.018	-	0.32384	0.59999	0.97015
Kaz/SW Rus	0.011	0.002	0.019	0.019	-0.000	0.017	-	0.54314	0.93078
Iceland	0.106*	0.088*	0.111*	0.094*	0.175*	0.134*	0.129*	-	0.90610
E Russia	-0.071	-0.061	-0.004	-0.040	-0.127	-0.042	-0.093	0.081	-

Results from STRUCTURE strongly supported a scenario with four genetic clusters, three within *Limosa limosa* and one comprising *Limosa haemastica*. The maximum average log likelihood Ln P(X|K) showed a maximum at K= 4 (Figure 2). Birds from Iceland (*L. l. islandica*) were assigned to a

separate cluster. Birds from the breeding range of *L. l. limosa* were assigned to two different clusters, hereafter clusters 1 and 2 (Figure 2). Birds from The Netherlands were assigned to cluster 1 only. Assignment of the other *L. l. limosa* populations was more ambiguous, with individuals and populations being assigned to either cluster 1 or both clusters 1 and 2. Only in the Swedish populations did assignment values for cluster 2 exceed 60%. Eastern Russian birds (*L. l. melanuroides*) were not recognized as a distinct genetic entity, but clustered together with birds from *L. l. limosa* breeding locations. Mantel tests detected significant correlation between genetic distance and geographic distance (P = 0.006), but not when Icelandic birds were excluded (P = 0.313).

Figure 2. Above: mean log likelihood Ln P(X|K) and DeltaK as a function of the number of genetic clusters (K) averaged over 5 consecutive STRUCTURE runs for each K (error bars indicate one standard deviation). Below: representation of the assignment values, estimated relative contribution of each member of the population to that individual's microsatellite-based genome, per individual at the different sample locations for K=4. Within *L. l. limosa* 2 clusters according to assignment values are depicted.

Recent gene flow estimates demonstrated migration from The Netherlands to all other sampling areas except Iceland (Table 4). Historic gene flow also showed high emigration rates from The Netherlands towards all other areas, including Iceland (Table 5).

Table 4. Bayesian estimates of recent gene flow among sample locations according to Bayesass. Migration is represented as the percentage of the total amount of migration towards a sample location with 95% confidence intervals. Rows represent emigration rates, columns immigration rates.

	Netherlands	M Germany	N Germany	Denmark	Belarus	Sweden	Kaz/W Rus	Iceland	E Russia
Netherlands	99.26 (97.80-99.92)	27.65 (21.01-32.22)	21.03 (10.51-30.39)	18.20 (8.04-29.44)	13.21 (3.43-27.24)	27.53 (20.84-31.70)	27.40 (20.10-32.39)	0.18 (0.00-1.55)	5.31 (0.32-16.21)
M Germany	0.04 (0.00-0.30)	68.05 (66.70-71.35)	1.17 (0.00-5.61)	1.28 (0.00-5.66)	1.77 (0.00-7.13)	0.27 (0.00-1.74)	0.49 (0.00-3.04)	0.12 (0.00-1.15)	2.43 (0.00-9.81)
N Germany	0.16 (0.00-0.73)	0.54 (0.00-3.00)	69.02 (66.73-75.09)	1.42 (0.00-6.90)	1.81 (0.00-7.90)	0.29 (0.00-1.77)	0.46 (0.00-2.59)	0.12 (0.00-0.98)	2.48 (0.00-10.32)
Denmark	0.05 (0.00-0.40)	0.49 (0.00-3.19)	1.26 (0.00-5.81)	69.28 (66.75-75.36)	1.71 (0.00-7.45)	0.28 (0.00-1.67)	0.49 (0.00-2.92)	0.14 (0.00-1.16)	2.39 (0.00-9.19)
Belarus	0.04 (0.00-0.34)	0.56 (0.00-3.31)	1.16 (0.00-5.65)	1.36 (0.00-6.33)	70.44 (66.80-78.95)	0.27 (0.00-1.62)	0.43 (0.00-2.57)	0.12 (0.00-1.04)	2.50 (0.00-10.63)
Sweden	0.09 (0.00-0.63)	0.56 (0.00-3.59)	1.54 (0.00-6.52)	3.29 (0.02-9.71)	3.42 (0.02-11.62)	69.97 (67.08-75.28)	1.08 (0.00-5.02)	0.16 (0.00-1.53)	2.44 (0.00-10.28)
Kaz/SW Rus	0.05 (0.00-0.39)	0.50 (0.00-2.85)	1.20 (0.00-5.95)	1.33 (0.00-6.19)	1.94 (0.00-8.01)	0.28 (0.00-1.77)	67.99 (66.71-71.26)	0.14 (0.00-1.33)	2.41 (0.00-9.49)
Iceland	0.19 (0.00-1.32)	0.61 (0.00-3.36)	1.35 (0.00-6.24)	1.40 (0.00-6.77)	1.93 (0.00-7.98)	0.57 (0.00-2.91)	0.63 (0.00-4.01)	98.74 (95.53-99.96)	2.59 (0.00-10.25)
E Russia	0.04 (0.00-0.34)	0.53 (0.00-3.17)	1.11 (0.00-5.88)	1.27 (0.00-5.98)	1.86 (0.00-7.82)	0.26 (0.00-1.72)	0.55 (0.00-3.23)	0.13 (0.00-1.09)	74.97 (67.00-90.68)

Table 5. Bayesian estimates of long-term gene flow among sample locations according to Migrate-n. All pairwise estimates of M (m/u) are shown with 95% confidence intervals. Rows represent emigration rates, columns immigration rates.

	Netherlands	M Germany	N Germany	Denmark	Belarus	Sweden	Kaz/W Russia	Iceland	E Russia
Netherlands	0	10.122 (7.400-12.800)	5.000 (2.267-7.667)	4.778 (2.533-7.000)	3.694 (0.667-6.667)	15.531 (13.267-17.733)	9.193 (4.733-12.867)	10.566 (7.867-13.133)	2.411 (0.133-4.667)
M Germany	2.212 (0.333-4.067)	0	1.325 (0.000-3.267)	1.150 (0.000-3.000)	0.541 (0.000-2.133)	1.764 (0.000-3.533)	1.561 (0.000-3.333)	2.212 (0.200-4.133)	1.121 (0.000-2.933)
N Germany	1.448 (0.000-3.067)	1.257 (0.000-3.067)	0	0.602 (0.000-2.267)	0.951 (0.000-2.733)	1.220 (0.000-2.933)	1.215 (0.000-3.000)	0.921 (0.000-2.667)	0.718 (0.000-2.400)
Denmark	1.367 (0.000-3.000)	0.672 (0.000-2.400)	0.937 (0.000-2.733)	0	0.556 (0.000-2.133)	0.572 (0.000-2.200)	1.783 (0.000-3.533)	0.976 (0.000-2.733)	1.076 (0.000-2.867)
Belarus	0.807 (0.000-2.467)	0.576 (0.000-2.200)	0.905 (0.000-2.667)	0.499 (0.000-2.067)	0	1.057 (0.000-2.800)	1.094 (0.000-2.867)	0.471 (0.000-2.000)	0.571 (0.000-2.200)
Sweden	4.023 (1.933-6.067)	2.811 (0.400-5.200)	1.450 (0.000-3.600)	1.858 (0.000-3.800)	1.017 (0.000-2.867)	0	3.469 (1.000-5.867)	2.190 (0.067-4.133)	0.712 (0.000-2.400)
Kaz/SW Russia	2.133 (0.267-3.933)	1.547 (0.000-3.467)	0.874 (0.000-2.600)	1.320 (0.000-3.067)	0.578 (0.000-2.133)	2.166 (0.200-4.067)	0	0.756 (0.000-2.467)	1.140 (0.000-2.933)
Iceland	2.350 (0.467-4.200)	1.971 (0.000-3.867)	1.209 (0.000-3.000)	1.507 (0.000-3.267)	1.484 (0.000-3.467)	2.475 (0.467-4.467)	2.314 (0.133-4.400)	0	0.301 (0.000-1.733)
E Russia	0.635 (0.000-2.267)	0.792 (0.000-2.533)	0.591 (0.000-2.200)	0.659 (0.000-2.333)	0.447 (0.000-1.933)	0.883 (0.000-2.600)	0.966 (0.000-2.733)	0.497 (0.000-2.067)	0

Mitochondrial analysis (mtDNA)

The subset of COI barcode sequences from our dataset was combined with the public *Limosa* sequences on BOLD as well as several *Limnodromus scolopaceus* and *Limnodromus griseus* sequences as outgroup (Figure 3). According to previous phylogenetic studies (Thomas *et al.* 2004), *Limnodromus* is the closest sister genus of *Limosa*. Genetic distances between COI barcodes have been shown to be a good indicator of phylogenetic relationships (Wilson *et al.* 2011). In the COI Neighbor-Joining tree, the clade containing *L. haemastica* and *L. fedoa* was the nearest sister to *L. limosa*, with 8.3% and 8.5% pairwise distance to each species, respectively.

This makes them both appropriate as outgroup for the HVR phylogenetic analysis. *L. lapponica* was placed as sister to the above, with 10.4% pairwise distance to *L. limosa*. Within *L. l. limosa*, COI sequences were 100% identical for 57 individuals from samples throughout the breeding distribution of *L. l. limosa*. COI sequences were derived from different PCR batches, with samples from diverse sources including blood, eggshell and muscle tissue, from which DNA was extracted by different people and in different laboratory rooms. Moreover, all the public BOLD sequences also consisted of this most common haplotype. Lack of variation due to large-scale contamination issues can thus be ruled out. *L. l. islandica* sequences were placed within the *L. l. limosa* cluster, distinguished by a single diagnostic character. Our *L. l. melanuroides* sequences as well as several BOLD sequences formed a monophyletic sister cluster to *L. l. limosa* and *L. l. islandica*, with minimally 2.0% pairwise distance. However, five BOLD sequences of specimens from the distribution range of *L. l. melanuroides* contained COI haplotypes that differed at a single position from the most common *L. l. limosa* haplotype and fell within that cluster. Some of these specimens were collected in Vietnam and could therefore not be linked to a specific breeding location. However, two were collected at the Selenga river delta area, the same location as our *L. l. melanuroides* samples and a known *L. l. melanuroides* breeding area.

Nucleotide diversity (pi), haplotype diversity (h) and number of haplotypes (nh) are summarized in Table 2. A total of 37 different haplotypes are found within the HVR 1 and 2 dataset. Phylogenetic trees of mitochondrial HVR 1 and 2 derived from Bayesian and Maximum Likelihood analysis are shown in a Bayesian tree (Figure 4). Where branch topology agree between the Bayesian and Maximum likelihood analysis, both support values are displayed on the respective Bayesian tree branches. As with the COI barcode data, both analyses support two monophyletic clades: one containing the individuals from Eastern Russia (PP > 0.95; bootstrap value > 80), the other containing all other individuals (PP > 95%; bootstrap value > 80%). The resolution of the HVR data was much greater than that of COI barcode, however. All Icelandic samples but one were recovered on a monophyletic sister clade to the *L. l. limosa* clade, while a single sample from Iceland (H072) fell within the *L. l. limosa* clade, making *L. l. limosa* and *L. l. islandica* paraphyletic.

Next page:
Figure 3. Neighbor-joining tree based on COI barcode mitochondrial sequences of *Limosa* with *Limnodromus* as outgroup (Thomas *et al.* 2004). Aside from the barcode sequences generated for this study, public sequences for *Limosa haemastica*, *Limosa fedoa*, *Limosa lapponica*, *Limnodromus scolopaceus* and *Limnodromus griseus* available through BOLD were included as well, indicated by their BOLD ID.

Neighbor-joining tree of the genus
Limosa COI sequence
625bp

E04_Lim_lim_CD041_COI
F029_Lim_lim_CD041_COI
H041_Lim_lim_CD041_COI
E66_Lim_lim_CD041_COI
BROM203-06|Limosa_limosa|COI-5P
H222_Lim_lim_CD041_COI
25926517_Limosa_limosa_limosa
F72_Lim_lim_CD041_COI
F001_Lim_lim_CD041_COI
F70_Lim_lim_CD041_COI
F69_Lim_lim_CD041_COI
F004_Lim_lim_CD041_COI
H040_Lim_lim_CD041_COI
F71_Lim_lim_CD041_COI
F39_Lim_lim_CD041_COI
H204_Lim_lim_CD041_COI
H210_Lim_lim_CD041_COI
H138_Lim_lim_CD041_COI
H056_Lim_lim_CD041_COI
BROM817-07|Limosa_limosa|COI-5P
H157_Lim_lim_CD041_COI
H135_Lim_lim_CD041_COI
H137_Lim_lim_CD041_COI
H197_Lim_lim_CD041_COI
E67_Lim_lim_CD041_COI
C22_Lim_lim_CD041_COI
C70_Lim_lim_CD041_COI
25924532_Limosa_limosa_limosa
25925090_Limosa_limosa_limosa
BROM818-07|Limosa_limosa|COI-5P
H195_Lim_lim_CD041_COI
H158_Lim_lim_CD041_COI
F002_Lim_lim_CD041_COI
25924550_Limosa_limosa_limosa
SWEBI020-11|Limosa_limosa|COI-5P
F41_Lim_lim_CD041_COI
C21_Lim_lim_CD041_COI
B39_Lim_lim_CD041_COI
25926973_Limosa_limosa_limosa
H221_Lim_lim_CD041_COI
25924541_Limosa_limosa_limosa
B04_Lim_lim_CD041_COI
BROM204-06|Limosa_limosa|COI-5P
B02_Lim_lim_CD041_COI
F030_Lim_lim_CD041_COI
F033_Lim_lim_CD041_COI
F40_Lim_lim_CD041_COI
F56_Lim_lim_CD041_COI
F58_Lim_lim_CD041_COI
F032_Lim_lim_CD041_COI
F42_Lim_lim_CD041_COI
H220_Lim_lim_CD041_COI
F43_Lim_lim_CD041_COI
F54_Lim_lim_CD041_COI
H203_Lim_lim_CD041_COI
H206_Lim_lim_CD041_COI
F53_Lim_lim_CD041_COI

L. l. limosa

BROM215-06|Limosa_limosa|COI-5P
BROM822-07|Limosa_limosa|COI-5P
KBPBU780-06|UWBM_73771|Limosa_limosa|
BROM906-08|Limosa_limosa|COI-5P
KBPBU780-06|Limosa_limosa|COI-5P|GQ482062

L. l. melanuroides

H073_Lim_isl_CD041_COI
BROM196-06|Limosa_limosa|COI-5P
H085_Lim_isl_CD041_COI
H088_Lim_isl_CD041_COI
BROM905-08|Limosa_limosa|COI-5P
BROM819-07|Limosa_limosa|COI-5P
BROM195-06|Limosa_limosa|COI-5P

L. l. islandica

E19_Lim_lim_CD041_COI
E26_Lim_lim_CD041_COI
F005_Lim_lim_CD041_COI
B68_Lim_lim_CD041_COI
H044_Lim_lim_CD041_COI

L. l. limosa

BROM248-06|Limosa_limosa|COI-5P
KKBNA728-05|Limosa_limosa|COI-5P|GQ482066
KBPBU779-06|Limosa_limosa|COI-5P|GQ482064
BROM823-07|Limosa_limosa|COI-5P
BROM821-07|Limosa_limosa|COI-5P
BROM820-07|Limosa_limosa|COI-5P
H109_Lim_mel_CD041_COI
BROM907-08|Limosa_limosa|COI-5P
KKBNA290-05|UWBM_46884|Limosa_limosa|
KKBNA728-05|UWBM_47118|Limosa_limosa|
KBPBU779-06|UWBM_73550|Limosa_limosa|
KKBNA290-05|Limosa_limosa|COI-5P|GQ482063
KBPBU152-06|UWBM_46888|Limosa_limosa|
KBPBU152-06|Limosa_limosa|COI-5P|GQ482065

L. l. melanuroides

Limosa limosa

TZBNA120-03|Limosa_haemastica|COI-5P|AY666318
TZBNA120-03|JAD_7512|Limosa_haemastica|
BROM428-06|Limosa_haemastica|COI-5P
BROM815-07|Limosa_haemastica|COI-5P
BROM267-06|Limosa_haemastica|COI-5P
BROM268-06|Limosa_haemastica|COI-5P
BROM900-08|Limosa_haemastica|COI-5P
TZBNA112-03|JAD_7254|Limosa_haemastica|
BROM173-06|Limosa_haemastica|COI-5P
BROM175-06|Limosa_haemastica|COI-5P
TZBNA112-03|Limosa_haemastica|COI-5P|AY666302
BROM899-08|Limosa_haemastica|COI-5P
H145_Lim_hea_CD041_COI

Limosa haemastica

Limosa

BROM898-08|Limosa_fedoa|COI-5P
BROM176-06|Limosa_fedoa|COI-5P
TZBNA094-03|Limosa_fedoa|COI-5P|AY666327
TZBNA103-03|Limosa_fedoa|COI-5P|AY666307
BROM172-06|Limosa_fedoa|COI-5P
TZBNA103-03|RCA_87-263|Limosa_fedoa|
TZBNA094-03|JGS_1740|Limosa_fedoa|
KKBNA160-04|CWS3840|Limosa_fedoa|
KKBNA160-04|Limosa_fedoa|COI-5P|DQ433773

Limosa fedoa

Limosa lapponica

Limosa lapponica
Limnodromus scolopaceus
Limnodromus griseus

0.01

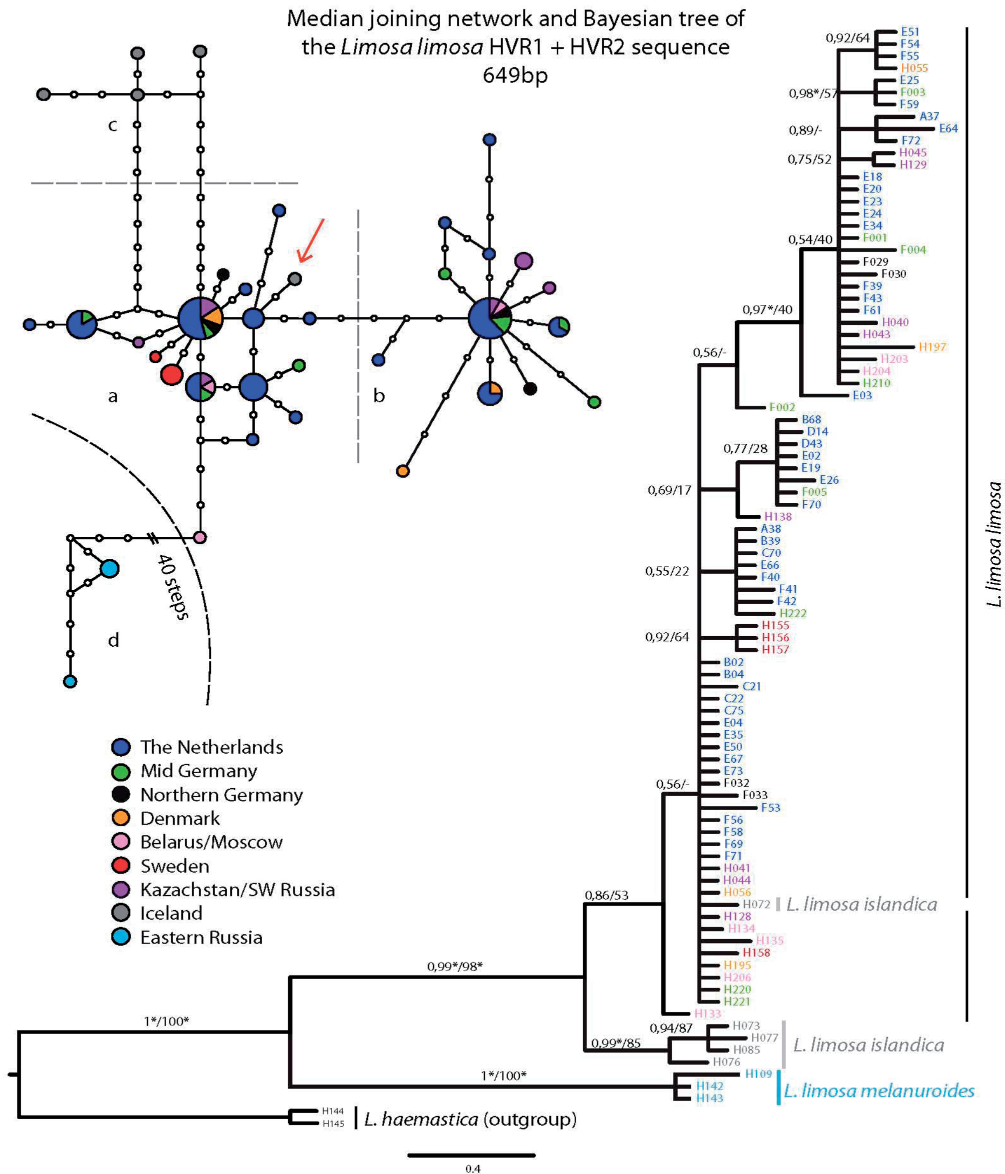


Figure 4. Analysis of the mitochondrial HVR1 and HVR2 for the three *Limosa limosa* subspecies. The colors indicate the sample locations. The support values of the Bayesian (left-hand value) and maximum likelihood (right-hand value) analysis are plotted on the Bayesian tree. Significantly supported (0.95 or higher) branches are indicated with a *. Additionally, a median-joining network of 89 HVR1 and HVR2 mtDNA sequences is depicted. Different clusters are indicated with a/b/c/d. The red arrow indicates one *L. l. islandica* individual (H072) that sorted close to *L. l. limosa* haplotypes.

A split in the *L. l. limosa* clade was significantly supported by the Bayesian analysis. However, these two genetic clusters did not correspond to geographically separated populations.

A median joining network based on the mitochondrial HVR1 and HVR2 sequences is shown in Figure 4. The basic structure of the network strongly resembles the phylogenetic trees. Haplotypes of the individuals from Eastern Russia (d) are separated from all others (abc) by at least 45 steps (Figure 4). Four Icelandic samples (c) are grouped together but separated from sample locations within the *L. l. limosa* breeding range (ab) by at least 11 steps, while one Icelandic sample is found within the *L. l. limosa* cluster (red arrow in Figure 4). The individuals from the *L. l. limosa* breeding locations group into two star-shaped clusters (a and b), with the most common haplotypes separated by eight steps. The two star-shaped clusters do not correspond to geographically separated populations (Figure 4). Swedish *L. l. limosa* individuals belong to cluster a, but display two unique haplotypes. These results are supported by Φst calculations, which showed higher values for pairwise differences between Eastern Russian and all other individuals (Φst values between 0.91-0.97) as compared with pairwise differences between Icelandic and other individuals (Φst values between 0.44-0.54). Φst values between Sweden and other sample locations are moderate (Φst values between 0.24-0.40). Neither Fu's Fs (ranging from -9.47 to 1.61, $P > 0.50$) nor Tajima's D (ranging from -1.32 to 1.32, $P > 0.10$) nor Fu and Li's D* (ranging from -1.01 to 1.29, $P > 0.10$) and F* (ranging from -0.95 to 1.32, $P > 0.10$) are significant for the total population or any of the sampling locations.

Discussion

Three subspecies have been recognized morphologically within *Limosa limosa* (*L. l. limosa*, *L. l. islandica* and *L. l. melanuroides*) and have been confirmed to be genetically identifiable as well in a previous study using the 'conserved domain' of the mitochondrial CR (Höglund *et al.* 2009). Here we confirm this distinction. Nevertheless, the signals found in the nuDNA did not support the split between *L. l. melanuroides* and *L. l. limosa* demonstrated by the mtDNA.

Nuclear DNA showed significant heterozygote deficiency in the Netherlands. MICROCHECKER analysis showed no signs of null alleles within this population, indicating that heterozygote deficiency was not an effect of null alleles. It is also unlikely that it was caused by a Wahlund effect (Wahlund 1928). As previous population genetic research could not detect any genetic population structure among Black-tailed Godwits breeding in different areas in The Netherlands (Trimbos *et al.* 2011), a possible explanation could be that there are few migration events from other locations towards The Netherlands (note recent gene flow analysis and significant F_{IS} value, Table 2). The nuDNA data demonstrate genetic differentiation between *L. l. islandica* on the one hand and *L. l. limosa* and *L. l. melanuroides* on the other. However, no genetic divergence was detected between *L. l. melanuroides* and *L. l. limosa*. Furthermore, within *L. l. limosa*, individuals from The Netherlands and Sweden appear to be divided into two distinct clusters. Individuals from other *L. l. limosa* breeding areas show an admixture of genotypes between the two clusters.

Only three COI barcode haplotypes were found within *L. l. limosa*, 92% of all samples of which contained the same haplotype. The other two subspecies contained only a single haplotype each. The lack of subspecific variation in COI barcode has been noted for other bird species, too, with various explanations being proferred, including selective sweeps or genetic drift through population bottlenecks (Kerr *et al.* 2007). This is probably an artefact of the lower substitution rate in COI compared to the HVR regions of the mtDNA (Buehler and Baker 2005, Wenink and Baker 1996). Even though the resolution exhibited by the COI barcode is less than the resolution of the HVR1 and HVR2 data, the subspecies are distinguishable by both parts of the mtDNA. Interestingly, all three mtDNA regions (COI, HVR1 and HVR2) show genetic differentiation between *L. l. melanuroides* and *L. l. limosa* individuals to be much higher than that between *L. l. limosa* and *L. l. islandica* individuals. Again interestingly, a single individual from Iceland (H072) contains a combined HVR1/HVR2 haplotype that closely resembles that of *L. l. limosa* individuals. To confirm that this was not due to contamination, we re-examined the microsatellite results from this extract. The microsatellite pattern of H072 was unique and contamination of the extract was thus ruled out; the lowest genetic distance found in all pairwise comparisons with H072 was 8 differences. Furthermore, we repeated the HVR PCR and sequencing for this sample twice, with no change in the results. Our results therefore indicate that there might have been recent hybridization events between *L. l. limosa* and *L. l. islandica*. *L. l. limosa* is divided into two large star-like haplotype clusters in the HVR data. These clusters are not supported geographically, as both haplotype clusters are present at nearly all the *L. l. limosa* sample locations. The two *L. l. limosa* haplotype clusters in the HVR mtDNA (Figure 4; cluster a and b) do not completely correspond with the *L. l. limosa* clusters found in the nuDNA (Figure 2; cluster 1 and 2). The two nuDNA clusters in *L. l. limosa* are comprised of the Netherlands, Northern Germany, Denmark, Belarus and Kazachstan/Russia, which make up cluster 1, and Sweden (over 60% assigned), which makes up cluster 2. Mid-Germany shows an almost even admixture between both clusters. So, while the differentiation between *L. l. limosa* and *L. l. islandica* shows similar patterns in mtDNA and nuDNA, differentiation between *L. l. limosa* and *L. l. melanuroides* seems to show opposite patterns in the mtDNA and nuDNA.

Within the mtDNA private haplotypes in *L. l. islandica* do not support a scenario of mitochondrial gene flow between *L. l. limosa* and *L. l. islandica*, but the similarity between *L. l. islandica* and *L. l. limosa* haplotypes might indicate past mtDNA introgression. Indeed, the nuDNA data support historical rather than recent gene flow between *L. l. limosa* and *L. l. islandica*. Furthermore, *L. l. islandica* does not possess private alleles but differs from *L. l. limosa* only by its allele frequencies. Together, the nuDNA and mtDNA thus suggest relatively recent separation of *L. l. islandica* and *L. l. limosa*. The difference between the mtDNA and the nuDNA in regards to the differentiation between *L. l. melanuroides* and *L. l. limosa* was unexpected. The mtDNA exhibited a sharp divergence between *L. l. melanuroides* and the remaining Black-tailed Godwits, while in the nuDNA there was a lack of divergence between *L. l. melanuroides* and the *L. l. limosa*. It is known that programs like STRUCTURE are very conservative in assigning samples from a certain group to a cluster when the sample sizes of such a group is small or sampling scheme is biased (Schwartz and McKelvey 2009). Therefore, these results can most likely be explained by the low sample size of the *L. l. melanuroides* group which have probably biased microsatellite analysis conservatively in regards to the split between *L. l. melanuroides* en *L. l. limosa*. Alternatively, the combined nuDNA and mtDNA

results might suggest a distant split between *L. l. melanuroides* and the other Black-tailed Godwits, with recent gene flow between *L. l. melanuroides* and *L. l. limosa*. Other studies have explained discordant patterns between nuDNA and mtDNA through introgression events (Daly-Engel *et al.* 2012, Hefti-Gautschi *et al.* 2009, Jones *et al.* 2005, Zarza *et al.* 2011). It should be noted, though, that this scenario with respect to *L. l. melanuroides* and *L. l. limosa* would only hold for the *L. l. melanuroides* breeding population at the Selanga River Delta; other *L. l. melanuroides* breeding populations might still be isolated from *L. l. limosa*. Interestingly, this study and another recent study both show the presence of two COI *L. l. melanuroides* haplotype groups, one sorting close to *L. l. limosa* and the other showing more distinct divergence from *L. l. limosa*, at the Selanga River Delta area (Elbourne 2011). These groups might constitute two disjunct *L. l. melanuroides* breeding colonies. An alternate hypothesis would be the presence of a slightly diverged *L. l. limosa* breeding population and a *L. l. melanuroides* breeding population. This would suggest current overlap in *L. l. limosa* and *L. l. melanuroides* breeding sites, making a gene flow scenario between *L. l. limosa* and *L. l. melanuroides*, as proposed here, more likely.

Wenink and Baker (1996) and Buehler and Baker (2005) estimated the mutation rates for HVR1 and HVR2 at around 10% per Myr. For a sequence length of 649 bp this would translate to 6.4 x 10^{-5} mutations per year, with a range of 3.2 x 10^{-5} to 9.6 x 10^{-5}. This results in split estimates of approximately 347 (±174) Ky for *L. l. limosa* vs. *L. l. melanuroides*, 85 (± 43) Ky for *L. l. limosa* vs. *L. l. islandica* and 62 (± 31) Ky for the two mtDNA *L. l. limosa* clusters. This would indicate that the mtDNA population structure, according to HVR1 and HVR2, arose during the Pleistocene. Other studies have also reported the origin of lineage diversity of several bird species to lie within the Pleistocene (Jones *et al*. 2005, Otvall *et al*. 2005, Ronka *et al*. 2008). Iceland was covered in ice during the Weichselien (occuring between 116Ky – 11,5Ky), making it unlikely that *L. l. islandica* (85Ky ago) colonized the island during that period (Adams 1997, Schmitt 2007, www.ngdc.noaa.gov. We hypothesize that the most recent common ancestor of *L. l. islandica* colonized Iceland after the Pleistocene (i.e. in the last 12Ky) and that since then genetic isolation and drift have resulted in the genetic differentiation observed between these subspecies today. Lineage diversification between *L. l. limosa* and *L. l. melanuroides* lineages could have occurred via separate southward or northward founder events. During the Pleistocene the ice sheets that dominated the landscape in Northern Europe and America were absent in large parts of far eastern Russia and there is strong evidence from Beringia and north-eastern Asia that several species of plant and animal survived the last glaciation at high altitudes (Adams 1997, Schmitt 2007, www.ngdc.noaa.gov. We suggest that the ancestral *L. l. melanuroides* became isolated from the remaining Black-tailed Godwit population in the Beringian refugium during periods of glacial cooling in the Pleistocene, resulting in the split in the mtDNA. After glacial conditions alleviated, *L. l. melanuroides* and *L. l. limosa* gene flow might have been regained, with introgression as a result. Northward founder events by two separate *L. l. limosa* lineages subsequently expanding throughout the current *L. l. limosa* breeding range followed by recent isolation and genetic drift could explain the two distinct star-shaped HVR mtDNA haplotype clusters for *L. l. limosa* (a and b). Similar patterns have been found in the Herring Gull *Larus argentatus* complex (Liebers *et al.* 2004). While the mtDNA demonstrated that haplotypes belonging to both cluster a and b were present in all sample locations, the nuDNA shows that *L. l. limosa* individuals from the Netherlands are assigned to cluster 1 only. As the HVR mtDNA shows that *L. l. limosa*

structure is more recent than the divergence between *L. l. islandica* and *L. l. limosa*, one explanation for the different *L. l. limosa* patterns in mtDNA and microsatellites might be incomplete lineage sorting in the microsatellites. Alternatively, the Dutch *L. l. limosa* population expansion that took place during the first half of the 20th century (Beintema *et al.* 1995, Haverschmidt 1963) may have caused introgression among Dutch *L. l. limosa* breeding locations. This could have resulted in the genetic homogenization of the Dutch *L. l. limosa* breeding population in the nuDNA. Additionally, this recent population expansion of Dutch *L. l. limosa* may have resulted in introgression between Dutch *L. l. limosa* individuals and individuals from other *L. l. limosa* breeding locations as well. This is supported by the recent gene flow estimates as well as by most *L. l. limosa* individuals being assigned to the Dutch *L. l. limosa* cluster (cluster 1). Some divergence between Sweden and other *L. l. limosa* sampling locations is shown by the microsatellites. While the Swedish *L. l. limosa* individuals do not share any mtDNA haplotypes with other *L. l. limosa* individuals, they are closely related to other *L. l. limosa* individuals, which might indicate recently restricted gene flow between Swedish *L. l. limosa* and other *L. l. limosa* individuals.

Our data confirm divergence between the three *Limosa limosa* subspecies (cf. Höglund *et al.* 2009). According to the patterns observed and their geographic separation, we propose that the three traditional subspecies should be managed as three separate units. We believe the most likely explanation for the genetic structure found in this study is post-Pleistocene geographical separation of *L. l. islandica* and a distant Pleistocene split of *L. l. melanuroides*. Possibly, *L. l. limosa* and *L. l. melanuroides* have regained secondary contact at Selanga River Delta recently. The two star-shaped haplotype clusters visible in the mtDNA of *L. l. limosa* are most likely the result of one or more successful *L. l. limosa* populations carrying two ancestral haplotypes expanding post-Pleistocene. *L. l. limosa* individuals from The Netherlands may have spread throughout the *L. l. limosa* breeding range, resulting in the two still slightly admixed clusters in the microsatellites. However, incomplete lineage sorting and homoplasy affecting the microsatellite analysis could not be ruled out in this case. Our data highlight the importance of using both nuDNA and mtDNA simultaneously when studying range-wide population genetic structure in birds.

Acknowledgements

Lida Kanters, David Kleijn, Rene Faber, Astrid Kant, Gerrit Gerritsen, Wim Tijsen, Dirk Tanger, Angela Helmecke, Heinrich Belting, Ole Thorup, Jacob Höglund helped collect blood and eggshell membrane samples. We thank Jos Hooijmeijer, Petra de Goeij, Pedro Lourenço and the rest of the University of Groningen Black-tailed Godwit team for their help in collecting eggshells and blood samples in southwest Fryslân and for sharing their blood samples collected at other Dutch Black-tailed Godwit breeding sites. Marco van der Velde arranged laboratory space for DNA extractions in Groningen. Dick Groenenberg, Frank Stokvis, Menno Schilthuizen, Pim Arntzen, Kees Roselaar and Ben Wielstra helped with the interpretation of the data. Finally, we are grateful to Nigel Harle of Gronsveld for brushing up our English.

References

Adams JM (1997) Global land environments since the last interglacial. Oak Ridge National Laboratory, TN, USA. http://www.esd.ornl.gov/projects/qen/nerc.html.

Ballard JW, Chernoff B, James AC (2002) Divergence of mitochondrial DNA is not corroborated by nuclear DNA, morphology or behavior in *Drosophila simulans*. Evolution, 56, 527-545.

Beerli P, Felsenstein J (1999) Maximum-likelihood estimation of migration rates and effective population numbers in two populations using a coalescent approach. Genetics, 152, 763-773.

Beerli P, Felsenstein J (2001) Maximum liklihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. Proceedings of the National Academy of Sciences of the United States of America, 98, 4563-4568.

Beintema A, Moedt O, Ellinger D (1995) Ecologische Atlas van de Nederlandse Weidevogels. Schuyt & Co, Haarlem

BirdLife International (2009) Species factsheet: *Limosa limosa*. Downloaded from http://www.birdlife.org on 29/1/2010

Birdlife International (2004) Birds in Europe: population estimates, trends and conservation status. Cambridge, UK.

Buehler DM, Baker AJ (2005) Population divergence time and historical demography in Red Knots and Dunlins. Condor, 107, 497-513.

Chao A, Jost L, Chiang SC, Jiang Y-H, Chazdon RL (2008) A two-stage probabilistic approach to multiple-community similarity indices. Biometrics, 64, 1178-1186.

Chiucchi JE, Gibbs HL (2010) Similarity of contemporary and historical gene flow among highly fragmented populations of an endangered rattlesnake. Molecular Ecology, 19, 5345-5358.

Cramp S, Simmons KEL (1982) *The Birds of the Western Palearctic*. Vol III. Oxford: Oxford University press.

Daly-Engel TS, Seraphin KD, Holland KN, Coffey JP, Nance HA, Toonen RJ, Bowen BW (2012) Global phylogeography with mixed-marker analysis reveals male-mediated dispersal in the endangered Scalloped Hammerhead Shark (*Sphyrna lewini*). PLOS One, 7, 1-11.

Dent EA (2009) STRUCTURE HARVESTER version 0.56.3 http://taylor0.biology.ucla.edu/struct_harvest/

Edwards S, Bensch S (2009) Looking forward or looking backwards in avian phylogeography? Molecular Ecology, 18, 2930-2933.

Elbourne R (2011) Masters of Science thesis: COI Barcoding of the Shorebirds: Rates of Evolution and the Identification of Species. Department of Ecology and Evolutionary Biology, Department of Toronto.

Estoup A, Jarne P, Cournuet JM (2002) Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. Molecular Ecology, 11, 1691-1604.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology, 14, 2611-2620.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, 1, 47-50.

Fu Y (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking, and background selection. Genetics, 147, 915-925.

Fu Y, Li WH (1993) Statistical tsts of neutrality of mutations. Genetics, 133, 693-709.

Gill JA, Langston RHW, Alves JA, Atkinson PW, Bocher P, Cidraes Vieira N, Chrockford NJ, Gelinaud G, Groen N, Gunnarsson TG, Hayhow B, Hooijmaijer J, Kentie R, Kleijn D, Lourenco P, Masero, JA, Meunier F, Potts P, Roodbergen M, Schekkerman H, Schroeder J, Wymenga E, Piersma T (2007) Contrasting trends in two black-tailed godwit populations: A review of causes and recommendations. Wader Study Group Bulletin, 114, 43-50.

Glabutz JC (2004) CONVERT: a user friendly program to reformat diploid genotypic data for commonly used population genetic software packages. Molecular Ecology Notes, 4, 309-310.

Goudet J (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. Journal of Heredity, 86, 485-486.

Gunnarsson TG, Potts PM, Gill JA, Croger RE, Gelinaud G, Atkinsson PW, Gardarsson A, Sutherland WJ (2005) Estimating population size in Icelandic Black-tailed Godwits Limosa limosa islandica by colour-marking. Bird Study, 52, 153-158.

Hall TA (1999) Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41, 95–98.

Haverschmidt F (1963) The black-tailed godwit. E.J. Brill. Leiden

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. PLOS Biology, 2, e312.

Hefti-Gautschi B, Pfunder M, Jenni L, Keller V, Ellegren H (2009) Identification of conservation units in the European Mergus merganser based on nuclear and mitochondrial DNA markers. Conservation Genetics, 10, 87-99.

Höglund J, Johansson T, Beintema A, Schekkerman H (2009) Phylogeography of the Black-tailed Godwit *Limosa limosa*: substructuring revealed by mtDNA control region sequences. Journal of Ornithology, 150,45-53.

Huelsenback JP, Ronquist F (2001) MR-BAYES: Bayesian inference of phylogenetic trees. Bioinformatics, 17, 754–755.

Jones KL, Krapu GL, Brrandt DA, Ashley MV (2005) Population genetic structure in migratory sandhill cranes and the role of Pleistocene glaciations. Molecular Ecology, 14, 2645-2657.

Jost L (2008) G_{ST} and its relative do not measure differentiation. Molecular Ecology, 17, 4015-4026.

Kerr KCR, Stoeckle MY, Dove CJ, Weigt LA, Francis CM, *et al.* (2007) Comprehensive DNA barcode coverage of North American birds. Molecular Ecology Notes, 7, 535-543.

Librado P, Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25, 1451-1452

Liebers D, Helbig AJ, de Knijff P (2001) Genetic differentiation and phylogeography of gulls in the *Larus cachinnans-fuscus* group (Aves: Charadriiformes). Molecular Ecology, 10, 2447-2462.

Liebers D, de Knijff P, Helbig AJ (2004) The herring gull complex is not a ring species. Proceedings of the Royal Society London B, 271, 893-901.

Mantooth, S. J. and Riddle, B. R. (2011) Molecular Biogeography: The Intersection between Geographic and Molecular Variation. Geography Compass, 5, 1–20.

Meirmans PG, Hedrick PW (2011) Assessing population structure: F_{ST} and related measures. Molecular Ecology Resources, 11, 5-18.

Moore WS (1995) Inferring phylogenies from mtDNA variation: mitochondrial gene trees versus nuclear-gene trees. Evolution, 49, 718-726.

Moritz C (1994) Defining 'Evolutionarily Significant Units'for conservation. *TREE*, 9, 373- 375.

Paton T, Haddrath O, Baker AJ (2002) Complete mitochondrial DNA genome sequences show that modern birds are not descended from transitional shorebirds. Proceedings of the Royal Society London B, 269, 839-846.

Peakal R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes, 6, 288-295.

Posada D, Crandall KA (1998) MODELTEST: Testing the model of DNA substitution. Bioinformatics, 14, 817–818.

Pritchard JK, Stphens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics, 155, 945-959.

Ottvall R, Höglund J, Bensch S, Larsson K (2005) Population differentiation in the redshank (Tringa tetanus) as revealed by mitochondrial DNA and amplified fragment length polymorphism markers. Conservation Genetics, 6, 321-331.

Qiagen (2003) DNeasy Tissue Handbook. Protocol for isolation of total DNA from animal tissues. pp.18-20, QIAGEN. Valencia, California, USA.

Rach J, DeSalle R, Sarkar IN, Schierwater B, Hadrys H (2008) Character-based DNA barcoding allows discrimination of genera, species and populations in Odonata. Proceedings of the Royal Society B-Biological Sciences, 275, 237-247.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity, 86, 248-249.

Richardson DS, Jury FL, Blaakmeer K, Komdeur J, Burke T (2001) Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). Molecular Ecology, 10, 2263-2273.

Roselaar CS, Gerritsen GJ (1991) Recognition of Icelandic Black-tailed Godwit and its occurrence in The Netherlands. Dutch Birding, 13, 128-135.

Rönkä A, Kvist L, Karvonen J, Koivula K, Pakanen VM, Schamel D, Tracy DM (2008) Population genetic structure in the Themminck's stint *Calidris temminckii*, with an emphasis on Fennoscandian populations. Conservation Genetics, 9, 29-37.

Rubinoff D, Holland BS (2005) Between Two Extremes: Mitochondrial DNA is neither the Panacea nor the Nemesis of Phylogenetic and Taxonomic Inference. Systematic Biology, 54, 952-961.

Rubinoff D, Cameron S, Will K (2006) A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. *Journal of Heredity,* 97, 581-594.

Ruokonen M, Kvist L (2002) Structure and evolution of the avian mitochondrial control region. Molecular Phylogenetics and Evolution, 23, 422-432.

Schekkerman H, Teunissen WA, Oosterveld E (2008) The effect of 'mosaic management' on the demography of black-tailed godwit *Limosa limosa* on farmland. Journal of Applied Ecology, 45, 1067-1075.

Schindel D, Stoeckle M, Milensky C, Trizna M, Schmidt B, Gebhard C, Graves G (2011) Project Description: DNA Barcodes of Bird Species in the National Museum of Natural History, Smithsonian Institution, USA. ZooKeys 152: 87-91. doi: 10.3897/zookeys.152.2473

Schmitt T (2007) Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. Frontiers in Zoology, 4, 1-13.

Schwartz MK, McKelvey KS (2009) Why sampling scheme matters: the effect of sampling scheme on landscape genetic results. Conservation Genetics, 10, 441-452.

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology Letters, 9, 615-629.

Srivathsan A, Meijer R (2011) On the inappropriate use of the Kimura-2-parameter (K2P) divergences in the DNA-barcoding literature. Cladistics, 28, 190-194.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics, 123, 585-595.

Thomas G H, Wills MA, Sze´kely T (2004) A supertree approach to shorebird phylogeny. BMC Evolution and Biology, 28, 1471-2148.

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.

Trimbos KB, Musters CJM, Verkuil YI, Kentie R, Piersma T, de Snoo GR (2011) No evident spatial genetic structuring in the rapidly declining Black-tailed Godwit *Limosa limosa limosa* in The Netherlands. Conservation Genetics, 12, 629-636.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes, 4, 535–538.

Verkuil YI, Trimbos K, Haddrath O, Baker AJ (2009) Characterization of polymorphic microsatellite DNA markers in the black-tailed godwit (*Limosa limosa: Aves*). Molecular Ecology Resources, 9, 1415-1418.

Wenink PW, Baker AJ (1996) Mitochondrial DNA lineages in composite flocks of migratory and wintering Dunlins (*Calidris alpina*). Auk, 113, 744-756.

Wilson GA, Rannala B (2003) Bayesian inference of recent migration rates using multilocus genotypes. Genetics, 163, 1177-1191.

Wilson JJ, Rougerie R, Schonfeld J, Janzen DH, Hallwachs W, Hajibabaei M, Kitching IJ, Haxaire J, Hebert PDN (2011) When species matches are unavailable are DNA barcodes correctly assigned to higher taxa? An assessment using sphingid moths. *BMC ecology*, 11, 1-14.

Zarza E, Reynoso VH, Emerson BC (2011) Discordant patterns of geographic variation between mitochondrial and microsatellite markers in the Mexican black iguana (Ctensosaura pectinata) in a contact zone. Journal of Biogeography, 38, 1394-1405.

Zink RM (2004) The role of subspecies in obscuring avian biological diversity and misleading conservation policy. Proceedings of the Royal Society B-Biological Sciences, 271, 561-564.

Zink RM, Barrowclough GF (2008) Mitochondrial DNA under siege in avian phylogeography. Molecular Ecology, 17, 2107-2121.

Zwarts L, Bijlsma RG, van der Kamp J, Wymenga E (2009) Living on the edge; wetlands and birds in a changing Sahel. KNNV Publishing Zeist.

CHAPTER

7

General Discussion

General discussion

In this thesis Black-tailed Godwits (*Limosa limosa limosa*) from a range of breeding areas were genetically compared at a regional (in southwestern Fryslân), national (among most prominent breeding areas in the Netherlands), and global scale (among breeding areas from across the entire breeding range of the species) to increase our understanding of the effects of habitat fragmentation and habitat quality on this species. In The Netherlands, the Black-tailed Godwit has evolved into an agriculture-following species. Unfortunately, increasing intensification of agricultural practices, such as early mowing of grasslands, conversion of grassland to cornfields, drainage of the meadows and high cattle densities have influenced habitat quality and quantity negatively. This has resulted in stark decreases in recruitment (Roodbergen *et al.* 2012, Schekkerman *et al.* 2008;2009).

With the loss of settlers and the loss of good breeding habitats, over the last decades Black-tailed Godwit breeding areas have become fragmented over much of the breeding range, yet little is still known about the influence of such habitat fragmentation. With the work in this thesis an attempt has been made to provide an insight in the effects of habitat fragmentation on the population dynamics of Black-tailed Godwit populations at different spatial scales and the long term.

1. Can eggshell membranes be used as a reliable DNA source in genetic research?

To conduct genetic research, DNA samples are needed. The most widely used DNA source for this kind of research has been blood samples. Blood samples usually provide clean DNA of high quality. However, birds of which blood samples need to be obtained, have to be captured which causes a certain amount of stress. Additionally, capturing enough birds is time consuming and requires specialists. Consequently, sampling blood for genetic studies could limit the geographical scale on which genetic studies can be done. A faster and less invasive method of DNA sampling would allow large-scale assessments of genetic diversity and genetic differentiation. Fragments of egg shell are often left in the nest after hatching, and are easily collected by volunteers. The inner membrane of egg shells grows veins with blood of the fetus during the later stages of development, which could provide the DNA needed for genetic research. However, egg shell membranes are prone to contamination with bacteria and or viruses, which could potentially degenerate the genetic information that can be obtained. Furthermore, cross contamination from other egg-shell membranes in the same nest could prove to be problematic. The aim here was to investigate the usefulness of eggshell membranes as a DNA source for genetic research of egg laying species like birds and reptiles, by addressing eggshell membrane DNA quality, degeneration and cross-contamination. To this end a comparison was made between DNA from eggshell membranes with blood-derived DNA samples from the same individuals. No degeneration, cross contamination or deteriorated DNA quality were detected. Even in total genotypic comparison of eggshell membrane DNA and blood sample DNA, using 11 microsatellite loci, neither degeneration nor cross-contamination was apparent. This research clearly illustrates that eggshell membranes can be used for population genetic research. Therefore, apart from the blood samples already collected in other studies, that we had excess to, egg shell membranes were also collected as a DNA source in the rest of this thesis. Through this method a higher geographical sampling coverage of the Black-tailed Godwit breeding areas was possible.

2. *What are the genetic differences between breeding populations on intensively managed agricultural grassland and extensively managed agricultural grassland?*

In a previous study it was suggested that intronic variation in the CHD1-Z gene (CHD1-Z*) of Black-tailed Godwits (*Limosa limosa limosa*) breeding in southwest Friesland, The Netherlands, was under selection or linked to another gene that was under selection (Schroeder *et al.* 2010). Individuals with the CHD1-Z* allele were found in high quality habitat in a higher frequency than individuals without the CHD1-Z* allele, which indicated genetic population structure correlated with habitat quality on a local scale. Furthermore, it was shown that the presence of CHD1-Z* correlated with fitness components. Here we re-examine these interesting correlations using a much expanded dataset (on 2088 birds from 2004-2010 rather than 284 birds from 2004-2007). The presence of the CHD1-Z* allele showed a tendency in adult females to lay their clutches earlier. Additionally, the occurrence of the CHD1-Z* allele resulted in chick body mass and return rates being higher. Chicks with the Z* allele that had hatched early in the breeding season were heavier at birth compared to chicks without the Z* allele and chicks with the Z* allele that had hatched late. However, individuals carrying the CDH1-Z* allele did not occur more on extensive agriculturally managed land compared to intensive agriculturally managed land. This research indicated that the CHD1-Z* allele correlated with fitness measurements in the chicks and probably in adult females, suggesting that there is some form of positive selection working on Black-tailed Godwit populations in southwestern Fryslân: female adults with the CHD1-Z* allele had a trend towards laying earlier nests, followed by possible CHD1-Z* chicks which had a higher return rate in general and body mass if hatching occurred early in the season (which indirectly reflects early laid nests). Such a mechanism can be explained by the following line of reasoning. If females lay earlier clutches, these nests would have a higher chance to survive mowing practices on agricultural land (which generally take place during the second half of May) (Kleijn *et al.* 2010).

One recent study showed that in general Black-tailed Godwits did not adjust their laying date to earlier dates (Musters *et al.* 2010). This could either indicate that there is no advantage for Black-tailed Godwits to lay their clutches early, or that they are somehow unable to advance their breeding schedule. However, other studies showed that Black-tailed Godwits do have higher survival if they lay their clutches earlier (Kleijn *et al.* 2010; Schroeder et al. 2012). If females have the ability to lay early clutches it seems these females (carrying the CHD1-Z* allele) would simultaneously produce chicks, if such a chick also carried the CHD1-Z* allele, with higher body mass and return rates. Chicks with high body mass would have a higher survival during the most critical stages of development, which is suggested to lead to higher survival in general (Arnold *et al.* 2006, Schekkerman *et al.* 2008; 2009, Schroeder *et al.* 2012). Musters *et al.* (2010) suggested that the reason for not shifting their lay date, might be that the peak of adult Black-tailed Godwit food (earthworms) abundance, did not change to earlier dates over time either. This would indeed deprive Black-tailed Godwit females of adjusting their lay date to earlier dates. So, while in general Black-tailed Godwits seem unable to shift breeding to an earlier date, it seems that indeed it gives a selective advantage to breed earlier. These results indicate the importance of early-laid clutches and chick body mass for chick recruitment and thus increased survival, which has been demonstrated in a growing number studies over the last decade (Kentie *et al.* 2013, Kleijn *et al.* 2010, Schekkerman *et al.* 2007;2008;2009, Schroeder *et al.* 2012)

No direct correlations could be found between the presence of CHD1-Z* and habitat quality. In other words no genetic differences could be observed between breeding populations on intensive agricultural grassland and extensive agricultural grassland. On the other hand it was shown that return rates of chicks that had hatched on extensively managed agricultural land were higher than chicks that had hatched on intensively managed agricultural land. Chick recruitment (from egg to adult breeding bird), which can be loosely translated to chick return rates, is shown to be the most important determinant of current Black-tailed Godwit population trends (Schekkerman *et al.* 2008, Roodbergen *et al.* 2008, Kentie *et al.* 2013). The finding presented here, in regards to habitat quality, highlights the importance of extensive agricultural grassland management for Black-tailed Godwit survival, which is supported by several recent studies (Groen *et al.* 2012, Kentie *et al.* 2013, Schekkerman *et al.* 2007;2008;2009). Moreover, recent research postulates that focusing habitat restoration efforts on well connected, large areas with a minimum size of 100 ha and that are still rich in Black-tailed Godwit breeding numbers should safeguard the Black-tailed Godwit population in the Netherlands on the long term (Teunissen *et al.* 2012).

3. What are the genetic differences between geographically isolated breeding populations in The Netherlands?

Besides selection and decreasing habitat quality other processes can disrupt viability of natural populations. Habitat fragmentation could in theory severely impair gene flow between neighboring breeding areas, which would in turn increase the effects of inbreeding and genetic drift in these fragmented breeding populations (Frankham 2010). However, the extent of this effect is highly dependent on the effective population sizes of these populations, the dispersal capabilities of the species and their breeding site faithfulness. The Black-tailed Godwit is a migratory bird flying thousands of kilometers from breeding area to winter area and back. As already mentioned in the introduction, Groen (1993) showed 90% of the adult breeding Black-tailed Godwits returning within 700 m of the previous nest site. Natal philopatry was demonstrated as well with 75% of the birds returning within 18 km of their previous hatching site (Groen 1993). These findings were further supported by Kruk et al. (1998) who showed first time breeders dispersed beyond 50 km in regards to their previous hatching site. However, since it is largely unknown what effective population sizes are and if gene flow occurs on even larger spatial scales, it is still unclear if and if so, at what geographical level habitat fragmentation influences Black-tailed Godwit breeding populations. Therefore we investigated genetic diversity at and genetic differentiation and gene flow between different Black-tailed Godwit breeding locations within the Netherlands on the basis of 12 microsatellites. The distance between breeding areas ranged from 7 to 135 kilometers, which was far beyond dispersal distances reported in demographic studies up till now (Groen 1993, Kentie *et al.* 2011, Kruk *et al.* 1998, Van den Brink *et al.* 2008). No genetic differences, with either F_{ST} or D analysis, could be detected and gene flow estimates were larger than "one migrant per generation" between breeding areas. These findings indicate that the dispersal abilities of the Black-tailed Godwit far exceed the 18 km maximum reported in demographic studies and possibly stretch beyond 135 kilometers. If this is indeed the case, most breeding areas in the Netherlands are still connected through dispersal of one or more individuals per generation.

However, incomplete lineage sorting could also explain the lack of genetic differences found between Black-tailed Godwit breeding areas. Lineage sorting in turn depends on effective population size and time since divergence took place (Zink and Barrowclough 2008). Since the geographical fragmentation currently observed between Black-tailed Godwit breeding areas in the Netherlands is believed to have started roughly 50 years ago, it is likely that lineage sorting is not complete for theoretical splits currently present between Godwit breeding areas. Effective population sizes can also influence lineage sorting. The larger the effective population in two geographically isolated populations the longer it will take for both lineages to sort i.e., become genetically distinct from each other (Frankham 2010, Zink and Barrowclough 2008). Usually, effective population size is smaller than the estimated population size. So, if the number of breeding pairs at the geographically isolated breeding areas in the Netherlands could be considered real population sizes, effective population sizes in these isolated areas would be small and thus lineage sorting would be fast. However, breeding areas that seem geographically isolated are not necessarily the relevant populations. For instance when a breeding area is made up out of three geographically isolated patches, but these three areas are still linked together through gene flow, then effective population size in the breeding area could actually be quite large, while estimated populations would indicate three small populations. Therefore, lineages might not have had enough time to sort due to effective population sizes being large at the different sampled Black-tailed Godwit breeding areas. Nevertheless, it is clear that fragmentation events have not impacted Black-tailed Godwit breeding areas in the Netherlands (on a scale of 7 to 135 km) to such an extent that they can be shown through genetic analysis. This demonstrates the high dispersal capabilities of the Black-tailed Godwit.

There is, however, another factor that might have been influencing the genetic patterns, or rather the lack thereof, observed. Effective population size at neutral loci, like the microsatellites used here, are frequently four times that of the mitochondrial locus, because of recombination and mitochondrial DNA being only maternal inherited in most cases (Frankham 2010). Therefore, mitochondrial DNA might detect recent splits for which nuclear loci did not have time to sort (Zink and Barrowclough 2008). So, in theory mitochondrial DNA might have picked up genetic differences between Dutch Black-tailed Godwit breeding areas. The scale of the study area, the effective population sizes of the sampled areas, and DNA marker used in this study could thus have limited our ability to detect genetic differences. Therefore, global scale investigations of genetic structure were performed using mitochondrial DNA and nuclear DNA (see below).

4. What are the genetic differences between breeding populations on a global scale?

Breeding areas were sampled at different parts of the Black-tailed Godwits entire breeding range, including breeding areas of the three morphologically recognized supspecies (*L. l. limosa, L. l. islandica* and *L. l. melanuroides)*. Population genetic structure and phylogeographic patterns were analyzed between these breeding areas using three different parts (COI, HVR1 and HVR2) of the mitochondrial locus (mtDNA) next to the 12 microsatellite loci (nuDNA). Since mitochondrial DNA was used here, which should be able to pick up smaller genetic differences than microsatellites due to their faster lineage sorting, we included many samples of the Dutch Black-tailed Godwit breeding areas

(Zink and Barrowclough 2008). In previous studies mtDNA sequence data showed minimal genetic divergence between the three subspecies and an absence of substructuring within *L. l. limosa*. Here, the nuDNA data suggested slight genetic differentiation between *L. l. limosa* from Sweden and the Netherlands with other *L. l. limosa* sample locations showing genetic admixture between Dutch and Swedish genotypes, between *L. l. limosa* and *L. l. islandica*, but not between *L. l. limosa* and *L. l. melanuroides*. The mtDNA also demonstrated a split between *L. l. limosa* and *L. l. islandica* and showed two *L. l. limosa* haplotype clusters that were not geographically isolated. This result also included Dutch Black-tailed Godwit breeding areas that were re-examined using mtDNA. So, while different mtDNA haplotypes were found within the Netherlands they did not correspond to a geographical structuring of the Black-tailed Godwit breeding areas, again supporting the notion that Dutch Black-tailed Godwit breeding areas are still connected genetically. However, the mtDNA data did not seem to be completely consistent with the nuDNA pattern as mtDNA did support a split between *L. l. melanuroides* and *L. l. limosa*/*L. l. islandica.* And while the *L. l. limosa* haplotype groups showed more genetic detail than the genotype *L. l. limosa* clusters in the nuDNA, the general *L. l. limosa* pattern was similar in both markers, with the exception of the genetic pattern represented by Dutch *L. l. limosa* individuals. The discordance between nuDNA and mtDNA genetic structure, in regards to the split between *L. l. melanuroides* and *L. l. limosa/L. l. islandica*, might be explained by contamination of the DNA, homoplasy, small sample size, introgression, incomplete lineage sorting or a combination thereof (Ballard *et al.* 2002, Zarza *et al.* 2011, Zink and Barrowclough 2008).

Within *L.l. limosa*, mitochondrial COI sequences were 100% identical for 57 individuals from samples throughout the *L. l. limosa* distribution, which could indicate contamination issues. However, COI sequences were derived from different PCR batches, with samples from diverse sources including blood, eggshell and muscle tissue, from which DNA was extracted by different people and in different laboratory rooms. Moreover, most public sequences of *L. l. limosa* that were also added to our dataset, resulting from sequence runs performed in different labs around the world and by different research groups, also consisted of this most common haplotype. Additionally, the same DNA isolates were used in the microsatellite and mitochondrial HVR analysis. These analyses did not show identical DNA sequences or microsatellite genotypes in *L. l. limosa* for these individuals comparable to the COI structure for the most common haplotype. In every single PCR, fragment length run and sequence run, for every single marker, negative controls were added and analyzed to further exclude contamination issues. No contamination was found in any of the analyses performed. Therefore contamination issues can be ruled out.

Additional analysis indicated the presence of homoplasy within our dataset. Homoplasy occurs when microsatellite alleles have identical fragment lengths that are not identical by descent (Selkoe and Toonen 2006). Although the presence of homoplasy might cause underestimation of population genetic structure, simulation studies suggest that any bias thus introduced will be only slight and will generally have much less effect on estimates of population differentiation than migration or genetic drift (Estoup *et al.* 2002, Selkoe and Toonen 2006). Moreover, the less distant splits between *L. l. islandica* and *L. l. limosa* and the haplotype groups within *L. l. limosa* were displayed by the mtDNA. It is therefore unlikely that homoplasy issues had a conservative effect on the *L. l. melanuroides* signal only.

The most likely reason for the discordance in the *L. l. melanuroides* signal between nuDNA and mtDNA is probably small sample size. Additional microsatellite analysis with pruned sets of three randomly chosen samples per location indicated that microsatellite analysis, including STRUCTURE, are conservative with regards to assigning samples to a different groups. For instance, when the *islandica* group was limited to 3 individuals no clear signal became visible using STRUCTURE. Alternatively the different *L. l. melanuroides* signals found might be explained through a scenario of isolation of *L. l. melanuroides* from *L. l. limosa* in Beringia during the Last Glacial Maximum, possibly followed by recent introgression (visible in the nuDNA only). Combined COI results of our *L. l. melanuroides* samples and previously analysed *L. l. melanuroides* samples, with some samples from the same sample location, showed two groups, one demonstrating divergence from *L. l. limosa* and while the other group sorted very close to *L. l. limosa* (Elbourne 2011). These groups might constitute two disjunct *L. l. melanuroides* breeding colonies. An alternate hypothesis would be the presence of a slightly diverged *L. l. limosa* breeding population and a *L. l. melanuroides* breeding population, suggesting current overlap in *L. l. limosa* and *L. l. melanuroides* breeding sites.

With respect to the other genetic signals found, the nuDNA and mtDNA suggested relatively recent separation of *L. l. islandica* and *L. l. limosa*. Additional analysis indicated that during the Pleistocene separation of *L. l. islandica* from *L. l. limosa* occurred, followed by colonization of Iceland by the *L. l. islandica* during the Holocene. Within *L. l. limosa* founder events followed by population expansion that took place during the Holocene explained the genetic patterns in the mtDNA nicely. Incomplete lineage sorting regarding the theoretically existing and relatively recent splits in the *L. l. limosa* could explain why genetic structure found on the basis of nuDNA was less prominent than the structure found on the basis of mtDNA. Alternatively, the Dutch *L. l. limosa* population expansion that took place during the first half of the 20th century (Beintema *et al.* 1995, Haverschmidt 1963) may have caused introgression among Dutch *L. l. limosa* breeding locations. This could have resulted in the genetic homogenization of the Dutch *L. l. limosa* breeding population in the nuDNA. Moreover, it appeared that this recent *L. l. limosa* population expansion has resulted in introgression between Dutch *L. l. limosa* individuals and individuals from other *L. l. limosa* breeding locations as well. Some divergence between Sweden and other *L. l. limosa* sampling locations was shown by the nuDNA. While the Swedish *L. l. limosa* individuals did not share any mtDNA genetic haplotypes with other *L. l. limosa* individuals, they are closely related to other *L. l. limosa* individuals, which might indicate recently restricted gene flow between Swedish *L. l. limosa* and other *L. l. limosa* individuals.

Final thoughts

The aim of this thesis was to use genetics to investigate long term population dynamic processes in Black-tailed Godwit populations resulting from increasing habitat fragmentation or isolation of different breeding habitat on three different spatial scales.

On a global scale my study confirmed the presence of three genetically distinct groups now recognized as subspecies. However, no clear genetic differences were found between *L. l. limosa* across

most of its current breeding range. Possibly, there is some genetic differentiation between breeding areas in the Netherlands and Sweden. In contrast to Dutch Godwit breeding numbers (35.000 breeding pairs), Sweden does not hold large numbers of breeding Godwits (100-250 breeding pairs) (Birdlife International 2004), and Swedish Black-tailed Godwits mainly breed on islands in the Baltic, on Öland and Götland.These populations are also in decline (Birdlife International 2004). So, Sweden, and especially the island of Götland, does seem to harbour genetic information in a very small vulnerable population not found in the Netherlands. If it is one's goal to maintain the full genetic diversity presently available (the whole 'evolutionary potential'), then conservation efforts at Swedish Black-tailed Godwit breeding sites should have priority.

While most research is currently focused on *L. l. limosa* (breeding population requirements, migration routes, and effective management strategies, Kentie *et al.* 2013, Kleijn *et al.* 2010, Lourenco *et al.* 2010, Musters *et al.* 2010, Roodbergen *et al.* 2012) and *L. l. islandica* (migration routes and understanding the ongoing population expansion, Gill *et al.* 2007, Gunnarsson *et al.* 2005;2006) the *L. l. melanuroides* as a subspecies, its breeding areas and its conservational challenges are still largely under exposed. Therefore, I suggest that more research should be done on this subspecies.

Genetic structure in the Netherlands was studied here because Black-tailed Godwit breeding areas seemed geographically fragmented and it was unclear if this had led to genetic separation and inbreeding as well. If genetic structure would have been found this would either have demonstrated isolation of different breeding areas, where genetic drift would have caused either inbreeding and genetic differentiation with other areas, or the presence of founder populations (Frankham 2010). In the case of genetic isolation as a result of habitat fragmentation, management efforts could focus on either maintaining these isolated breeding areas by enlarging them and keep them isolated or connect them directly or indirectly through corridors or translocation. The choice of management action depends on whether the mixing of populations is necessary, for instance when genetic diversity of several breeding populations is low and increasing genetic diversity would thus increase population evolutionary potential. Connecting breeding areas directly by enlarging them would probably be the best strategy in this case.

The Black-tailed Godwit is a species that needs high water tables which are predominantly found in herb rich grasslands. A recent study demonstrated higher densities of breeding Black-tailed Godwits in ´herb rich grasslands´ (Kentie *et al.* 2013). When other populations would harbour high genetic diversity, and connecting breeding areas is not necessarily acute, a long term management strategy could be to enlarge isolated breeding populations (for instance by increasing herb rich grassland habitat), with unique genetic profiles, in order to sustain the genetic diversity and evolutionary potential of the Dutch Black-tailed Godwit population as a whole.

However, all analysis done indicated that habitat fragmentation, although geographically visible, has not yet lead to genetic differences between Dutch breeding populations (not on a local or a national scale). Slatkin (1985, 1987) concluded that only one migrant per generation, which for Black-tailed Godwits is generally considered as 3 years, is needed to obscure any disruptive effects of genetic drift.

On the other hand, Mills and Allendorf (1996) suggest that this number should actually be larger than 1 in many natural populations and that the one migrant per generation rule should be considered as a minimum. Additionally, another study showed that the size of the recipient population(s) under study might also influence the number of migrants needed to avoid excessive inbreeding (Vucetich and Waite 2000). Groen (1993) showed 90% of the adult breeding birds returned within 700 m of the previous nest site. The same research demonstrated natal philopatry to be high as well with 75% of the birds returning within 18 km of their previous hatching site. Demographic research conducted in southwestern Fryslân indicates that around 8% of the adult population formerly captured in that research area disperses beyond 3 km in following years (Kentie *et al.* 2011). Additionally, natal philopatry showed that 5% of the young adults bred beyond 8 kilometers of their former hatching site. The furthest dispersal movement of a young adult within the study area was about 18 kilometers (Kentie *et al.* 2011). There are some colour-ringed birds that have been observed several times in different years at the migration stopover sites but are not reported back at the research area in Fryslân during the breeding season, which could indicate that breeding dispersal outside of the breeding area took place (personal communication with R. Kentie). Kruk *et al.* (1998) showed that on the basis of ring recovery data from the Dutch ringing center between 1900 and 1991 nearly 3% of the natal philopatry took place beyond 50 km. These findings indicate breeding dispersal indeed takes place beyond the borders of the geographically isolated breeding areas, making it likely that the minimum of one migrant per three years does indeed keep different Dutch Black-tailed Godwit breeding areas genetically connected. As such it seems that demographic research does support the idea of a single panmictic population in The Netherlands.

A recent report has studied the Black-tailed Godwit breeding areas ´Black-tailed Godwit core areas´ that have the highest chance of stable population trends, based on soil type, groundwater tables, openness and recent local population trends (Teunissen *et al.* 2012). The main idea is to focus management and financial efforts in these areas instead of trying to preserve all breeding areas including unsustainable and thus futureless areas. A pitfall could be that important genetic diversity might be lost through this way of management, which in turn might impair the species ability to adapt to anthropological and environmental changes (Frankham 2010). Our results indicate that this is not the case on a spatial scale at the size of the Netherlands. In a way the results in this thesis thus support the idea of the ´Black-tailed Godwit core areas´ (Teunissen *et al.* 2012). The most important factors influencing these potential ´core areas´ were demonstrated to be openness, mowing date and watertable (Teunissen *et al.* 2012). Furthermore ´herb rich grasslands´ are demonstrated to be very important for Black-tailed Godwit recruitment (Kentie *et al.* 2013). In general ´herb rich grasslands´ have late mowing dates which increase nest and chick survival directly (Kentie *et al.* 2013, Kleijn *et al.* 2010). Additionally, ‘herb rich grasslands’ have high water tables, important for the maintenance of Black-tailed Godwit breeding and foraging habitat (Kentie *et al.* 2013, Kleijn *et al.* 2010). Openness, which in some ´herb rich grasslands´ is also quite prominent, is shown to influence nest and chick survival by decreasing predation chance (van der Vliet *et al.* 2008). Our fitness correlation analysis indicated the importance of ´herb rich grassland´ also, and it was demonstrated in a recent study to be great habitat for chicks providing good foraging and hiding opportunities (Kentie *et al.* 2013). Thus most determinants of ´Black-tailed Godwit core areas´ also play an important role

in ´herb rich grasslands´. Therefore focusing management strategies on ´Black-tailed Godwit core areas´ could help with the realization of large scale ´herb rich grassland´ areas. This in turn could potentially increase Black-tailed Godwit recruitment in The Netherlands, which has been decreasing over the last decades (Kentie *et al.* 2013, Schekkerman *et al.* 2008).

Genetics for species conservation

Genetics can be a helpful and insightful tool to investigate population structure and thereby population dynamic processes. In the past decades, genetic approaches have become more efficient in answering ecological questions and therefore more widespread. As a result, the accompanying genetic markers such as allozymes, microsatellites and mitochondrial and nuclear DNA sequences have been used to estimate a wide variety of parameters such as migration rates, population size, kinship, genetic structure and more. Mitochondrial DNA markers and microsatellites have become the most widely used tools in genetic research. Both were used in the research making up this thesis. However, although most of the genetic studies have used and are still using one or both of these markers to study genetic patterns in natural populations (Liebers *et al.* 2004, Ottval *et al.* 2005, Paton *et al.* 2002, Ronka *et al.* 2008, Zink and Barrowclough 2008, Zarza *et al.* 2011), the shortcomings which inherit both markers might severely impair the conclusions that can be drawn for species conservation or in the field of conservation biology currently.

Mitochondrial DNA is present in most cells in high copy number, making it easy to isolate mtDNA, and is relatively rapid and inexpensive to sequence. After sequencing, a phylogenetic tree of mtDNA haplotypes (variable genotypes of the mtDNA), rooted with a closely related taxon, will reveal whether closely related haplotypes occur locally or throughout the range and form a picture of the genetic structure. However, several problems have been defined since this marker type started to be used for studies of genetic structure. First, the mtDNA is one locus, which means that every gene that lays on the mtDNA evolves in a linked fashion together with the other genes on the mtDNA, with only one gene tree as a result irrespective of the amount of genes one would sequence. This might not give an adequate representation of the genetic structure present at the genome level of an individual. In other words, even if many genes of the mtDNA are sequenced the sample size for that one individual will always be one. An additional concern with mtDNA is the fact that it is maternally inherited, which entails that the structure found only portrays the female lineage. If a population would maintain gene flow through males only, the genetic structure in the mtDNA could give a wrong representation of lineage history. A third problem is base substitutions on the same base, which could potentially mask deeper lineage diversification (Zink and Barrowclough 2008, Edwards and Bensch 2009). The usage of nuDNA, sequenced at multiple inlinked loci, or unlinked microsatellites can solve some of these problems. nuDNA is both maternally and paternally inherited. Furthermore, by sampling a large amount of unlinked nuclear loci, which is possible with nuDNA, one improves the large confidence intervals otherwise associated with mtDNA phylogeographic analysis. Although issues concerning homoplasy have not yet been empirically tested, homoplasy is believed to pose problems in nuDNA sequence analysis as well as in microsatellite analysis (Selkoe and Toonen 2006, Zink

and Barrowclough 2008). Additionally, nuDNA has a serious impairment compared to mtDNA when it comes to coalescent times. Due to the fact that nuDNA is inherited through both parents and recombines during mitosis lineage sorting on average takes 4 times, termed $4N_e$, longer for nuDNA than for mtDNA. In general this phenomenon results in mtDNA being able to portray signals of lineage diversification much quicker than nuDNA can (Zink and Barrowclough 2008).

With this in mind one should probably consider mtDNA gene trees as the backbone of a genetic structure or phylogeographic study. Subsequently, nuDNA can be used to confirm the mtDNA signal and make CI's in several analysis smaller. However, in that case one does not consider that the mtDNA signal itself (although being able to show changes in genetic structure more quickly) being one locus only, might not be the general representation of population genetic structure on a genome level (Edwards and Bensch 2009). Should one consider different randomly dispersed loci with long coalescence times as indicative of a genetic pattern in a population or is one locus that is maternally inherited and has much faster coalescence time respectfully, better? In essence one also needs to address the following question: should we always look for as much genetic structure as possible; does the genetic signal with the most structure always give the most thorough representation of the populations under study?

Luckily, new techniques are arising on the horizon, which might be able to overcome the shortcomings of mtDNA and nuDNA for conservation genetic research. Next generation sequencing rapidly generates huge amounts of sequence data in a very cost-effective way making it possible for molecular ecologists to get a genome wide representation of genetic structure in non-model organisms (Ekblom and Galindo 2011, Jakobsson *et al.* 2008, Novembre *et al.* 2008). This technique has the potential to enhance the scale and detail of population genetic research enormously and make it possible to answer questions concerning habitat fragmentation and quality very thoroughly. Only small amounts of DNA are needed for detailed genetic analysis, which is helpful in difficult to sample species. Additionally, where currently only a handful of genes or microsatellites are used to answer conservation related questions the rise of NGS enables the shift from conservation genetics to conservation genomics. Potentially hundreds or thousands of genes can be studied possibly involved in phenotypic variation or selection (Ekblom and Galindo 2011, Ouborg *et al.* 2010). DNA sections under selection, generally considered to show very fast lineage sorting, could additionally be used to study, for instance, recent habitat fragmentation. However, with the rise of this very promising technique new problems arise. How to store thousands of terra or even peta bytes in a cost-effective way, and what does all the data mean from an ecological or conservation point of view? It is clear we are not quite there yet. Future challenges most likely lay in bioinformatics and interpreting all the genomic data to answer ecological and conservation related questions. Surely, the next decades are going to show some very exciting changes in genomics and related fields such as conservation genetics.

References

Arnold JM, Hatch JJ, Nisbet ICT (2006) Effects of egg size, parental quality and hatch-date on growth and survival of Common Tern *Sterna hirundo* chicks. Ibis, 148, 98-105.

Ballard JW, Chernoff B, James AC (2002) Divergence of mitochondrial DNA is not corroborated by nuclear DNA, morphology or behavior in *Drosophila simulans*. Evolution, 56, 527-545.

Birdlife International (2004) Birds in Europe: population estimates, trends and conservation status. Cambridge, UK.

Beintema A, Moedt O, Ellinger D (1995) Ecologische Atlas van de Nederlandse Weidevogels. Schuyt & Co, Haarlem.

Edwards S, Bensch S (2009) Looking forward or looking backwards in avian phylogeography? Molecular Ecology, 18, 2930-2933.

Elbourne R (2011) Masters of Science thesis: COI Barcoding of the Shorebirds: Rates of Evolution and the Identification of Species. Department of Ecology and Evolutionary Biology, Department of Toronto.

Ekblom R, Galinod J (2011) Applications of next generation sequencing in molecular ecology of non-model organisms. Heredity, 107, 1-15.

Estoup A, Jarne P, Cournuet JM (2002) Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. Molecular Ecology, 11, 1691-1604.

Frankham R, Ballou JD, Briscoe DA (2010) Introduction to Conservation Genetics. Cambridge, UK.

Gill JA, Langston RHW, Alves JA, Atkinson PW, Bocher P, Cidraes Vieira N, Chrockford NJ, Gelinaud G, Groen N, Gunnarsson TG, Hayhow B, Hooijmaijer J, Kentie R, Kleijn D, Lourenco P, Masero, JA, Meunier F, Potts P, Roodbergen M, Schekkerman H, Schroeder J, Wymenga E, Piersma T (2007) Contrasting trends in two black-tailed godwit populations: A review of causes and recommendations. Wader Study Group Bulletin, 114, 43-50.

Groen NM (1993) Breeding site tenacity and natal philopatry in the Black-tailed Godwit. Ardea, 81, 107-113.

Groen NM, Kentie R, de Goeij P, Verheijen B., Hooijmeijer JCEW, Piersma T (2012) A modern landscape ecology of Black-tailed Godwits: habitat selection in southwest Friesland (The Netherlands). Ardea, 100, 19-28.

Gunnarsson TG, Gill JA, Atkinson PW, Gélinaud G, Potts PM, Croger RE, Gudmundsson GA, Appleton GF, Sutherland WJ (2006) Population-scale drivers of individual arrival times in migratory birds. Journal of Animal Ecology, 75, 1119-1127.

Gunnarsson TG, Potts PM, Gill JA, Croger RE, Gelinaud G, Atkinsson PW, Gardarsson A, Sutherland WJ (2005) Estimating population size in Icelandic Black-tailed Godwits Limosa limosa islandica by colour-marking. Bird Study, 52, 153-158.

Haverschmidt F (1963) The black-tailed godwit. E.J. Brill. Leiden

Jakobsson m, Scholz SW, Scheet P, Gibbs JR, van Liere JM, Fung HC *et al.* (2008) Genotype, haplotype and copy-number variation in worldwide human populations. Nature, 451, 998.

Kentie R, Hooijmeijer JCEW, Both C, Piersma T (2011) Weidevogels in ruimte en tijd 2007-2010. Eindrapport. Ministerie van LNV, Directe Kennis, Den Haag.

Kentie R, Hooijmeijer JCEW, Trimbos KB, Groen NM, Piersma T (2013) How dairy farming practices affect growth and survival of meadow bird chicks. Journal of Applied Ecology, 50, 243-251.

Kleijn D, Schekkerman H, Dimmers WJ, Kats RJM, Melman D, Teunissen WA (2010) Adverse effects of agricultural intensification and climate change on breeding habitat quality of Black-tailed Godwits *Limosa l. limosa* in the Netherlands. Ibis, 152, 475-486.

Kruk M, Noordervliet MAW, Ter Keurs WJ (1998) Natal philopatry in the black-tailed godwit *Limosa limosa* L. and its possible implications for conservation. Ringing and Migration, 19, 13–16.

Liebers D, de Knijff P, Helbig AJ (2004) The herring gull complex is not a ring species. Proceedings of the Royal Society London B, 271, 893-901.

Lourenço PM, Kenti R, Schroeder J, Alves JA, Groen NM, Hooijmeijer JCEW, Piersma T (2010) Phenology, stop-over dynamics and population size of migrating black-tailed godwits *Limosa limosa* at a key staging area, the Tejo and Sado rice plantations. Ardea, 98, 35-42.

Mills LS, Allendorf FW (1996) The one-migrant-per-generation rule in conservation and management. Conservation Biology, 10, 1509-1518.

Musters CJM, Snoo GR de, Keurs WJ ter (2010) Timing of the breeding season of Black-tailed Godwit *Limosa Limosa* and Northern Lapwing *Vanellus vanellus* in the Netherlands. Ardea, 98, 195-202.

Novembre J, Johnson T, Bryc K, Kutalik Z, Boyko AR, Auton A *et al.* (2008) Genes mirror geography within Europe. Nature, 456, 98.

Ouborg NJ, Pertoldi C, Loeschcke V, Bijlsma R, Hedrick PW (2010). Conservation genetics in transition to conservation genomics. Trends in Genetics, 26, 177-187.

Ottvall R, Höglund J, Bensch S, Larsson K (2005) Population differentiation in the redshank (*Tringa tetanus*) as revealed by mitochondrial DNA and amplified fragment length polymorphism markers. Conservation Genetics, 6, 321-331.

Paton T, Haddrath O, Baker AJ (2002) Complete mitochondrial DNA genome sequences show that modern birds are not descended from transitional shorebirds. Proceedings of the Royal Society London B, 269, 839-846.

Rönkä A, Kvist L, Karvonen J, Koivula K, Pakanen VM, Schamel D, Tracy DM (2008) Population genetic structure in the Temminck's stint *Calidris temminckii*, with an emphasis on Fennoscandian populations. Conservation Genetics, 9, 29-37.

Roodbergen M, van der Werf B, Hötker H (2012) Revealing the contributions of reproduction and survival to the Europe-wide decline in meadow birds: review and meta-analysis. Journal of Ornithology, 153, 53-74.

Schekkerman H, Beintema AJ (2007) Abundance of invertebrates and foraging success of Black-tailed Godwit *Limosa limosa* chicks in relation to agricultural grassland management. Ardea, 95, 39-54.

Schekkerman H, Teunissen WA, Oosterveld E (2008) The effect of 'mosaic management' on the demography of Black-tailed Godwit *Limosa limosa* on farmland. Journal of Applied Ecology, 45, 1067-1075.

Schekkerman H, Teunissen WA, Oosterveld E (2009) Mortality of Black-tailed Godwit *Limosa limosa* and Northern Lapwing *Vanallus vanellus* chicks in wet grasslands: influence of predation and agriculture. Journal of Ornithology, 150, 133-145.

Schroeder J, Kentie R, van der Velde M, Hooijmeijer JCEW, Both C, Haddrath O, Baker AJ, Piersma T (2010) Linking intronic polymorphism on the CHD1-Z gene with fitness correlates in Black-tailed Godwits *Limosa l. limosa*. Ibis, 152, 368-377.

Schroeder J, Piersma T, Groen NM, Hooijmeijer JCEW, Kentie R, Lourenço PM, Schekkerman H, Both C (2012) Reproductive timing and investment in relation to spring warming and advancing agricultural schedules. Journal of Ornithology, 153, 327-336.

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology Letters, 9, 615-629.

Slatkin M (1987) Gene flow and the geographic structure of natural populations. Science, 236, 787-792.

Slatkin M (1985) Gene flow in natural popualtions. Annual review of Ecology and systematics, 16, 393-430.

Teunissen W, Schotman A, Bruinzeel LW, ten Holt H, Oosterveld E, Sierdsema H, Wymenga E, Melman D (2012) Op naar kerngebieden voor weidevogels in Nederland. SOVON Vogelonderzoek Nederland Beek-Ubbergen, Alterra Wageningen UR, Altenburg & Wymenga ecologisch onderzoek bv. Feanwâlden. Alterra-rapport 2344, Sovon-rapport 2012/21, A&W-rapport 1799.

van der Vliet RE, Schuller E, Wassen MJ (2008) Avian predators in a meadow landscape: consequences of their occurrence for breeding open-area birds. Journal of Avian Biology, 39, 523-529.

van der Vliet RE, van Dijk J, Wassen MJ (2010) How different landscape elements limit the breeding habitat of meadow bird species. Ardea, 98, 203-209.

Vucetich JA, Waite TA (2001) Migration and inbreeding: the importance of recipient population size for genetic management. Conservation Genetics, 2, 167-171.

Zarza E, Reynoso VH, Emerson BC (2011) Discordant patterns of geographic variation between mitochondrial and microsatellite markers in the Mexican black iguana (*Ctensosaura pectinata*) in a contact zone. Journal of Biogeography, 38, 1394-1405.

Zink RM, Barrowclough GF (2008) Mitochondrial DNA under siege in avian phylogeography. Molecular Ecology, 17, 2107-2121.

Summary

When raised bogs were converted to wet grasslands and hay lands, for the purpose of dairy farming, the opportunistic Black-tailed Godwit shifted from their former breeding habitat to these newly formed culture lands. From 1900 on the Black-tailed Godwit was mostly present in these damp hay lands, which were the major part of The Netherlands back then. Black-tailed Godwits profited from the increasing usage of artificial fertilizer at these damp hay lands, which in turn increased food availability. Consequently, Black-tailed Godwit population numbers increased drastically in these habitats during the first half of the 20th century. However, increased agricultural intensification, urbanization and decrease of available grassland areal, led to a decrease in habitat quality and the fragmentation of suitable grassland, in turn decreasing Black-tailed Godwit recruitment since the late 1960s. Population numbers have declined ever since with an annual rate estimated at 5%. Different management strategies, such as nest protection, creating reserves and mosaic management have been formulated and adopted to halt these decreases by improving nest success and habitat quality. However, up till now it remains unclear if these measurements do indeed have the desired effect on habitat quality and in turn habitat fragmentation. Furthermore, it remains ambiguous if habitat fragmentation is affecting Black-tailed Godwit populations at all.

Habitat fragmentation and changes in habitat quality might affect the population dynamics of a species. For instance decreasing habitat quality might cause a species to adopt a different dispersal pattern, or a breeding population might get isolated as a result of habitat fragmentation. These kind of population dynamic changes might in turn affect the genetic pattern of the species. In this thesis genetics were used to investigate long term population dynamic processes in Black-tailed Godwit populations resulting from increasing habitat fragmentation on three different spatial scales. However, before genetic research could be conducted genetic markers and easy sampling methods were needed. Therefore, microsatellite markers were developed and the usefulness of egg shell membranes as a source of DNA was assessed.

In chapter 2 we used the developed microsatellite markers to test egg shell membranes as an alternative sampling method to blood samples in population genetic research. We showed that genetic information within eggshell membrane DNA in comparison to blood DNA was not affected 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 demonstrated that eggshell membranes can be used for population genetic research.

In chapter 3 we re-examined the findings of a previous study that demonstrated intronic variation in the CHD1-Z gene (Z*) in Black-tailed Godwits was correlated with habitat quality on a local scale. The previous study suggested that Black-tailed Godwit breeding populations were differentially structured at habitat of different quality as a result of positive selection pressures on Black-tailed Godwits breeding at habitat of high quality. We demonstrate that the presence of the Z* allele was not correlated with habitat quality. Other calculations on fitness correlates show that there might be a tendency towards female adults with the Z* allele laying earlier clutches than adult females without the Z* allele. Also the occurrence of the Z* allele was associated with a higher chick body mass and return rate. Interestingly, return rates of chicks that had hatched on herb-rich agricultural

land were higher than return rates of chicks that had hatched on intensively managed meadows, which demonstrates the importance of high quality habitat for breeding Black-tailed Godwits.

In chapter 4 and 5 we investigated if Black-tailed Godwit breeding populations were genetically structured as a result of habitat fragmentation on the scale of The Netherlands. We demonstrated that genetic diversity was moderate and no genetic clustering was visible between Dutch Black-tailed Godwit breeding populations. Gene flow estimates were larger than “one migrant per generation” between sample locations, and no isolation by distance effect was demonstrated. These findings indicated the maintenance of moderate levels of genetic diversity throughout the Dutch Black-tailed Godwit population through appropriate levels of gene flow between different breeding areas. Dutch Black-tailed Godwit breeding areas did not seem to be affected genetically by habitat fragmentation.

In chapter 6, we expanded our focus on the effects of habitat fragmentation to a global scale. The genetic population structure and phylogeny of the Black-tailed Godwit was assessed entailing DNA samples ranging from Iceland to Lake Baikal in eastern Russia and including all three currently known subspecies of Black-tailed Godwit. A historic explanation was given for the genetic patterns found in both microsatellites and newly developed mtDNA markers. We demonstrated only very marginal patterns of genetic structure within the *L. l. limosa.* Furthermore genetic differentiation between subspecies *L. l. limosa* and *L. l. islandica*, but not between *L. l. limosa* and *L. l. melanuroides was* shown. However, the genetic patterns in the mtDNA seemed inconsistent with the nuDNA pattern. The mitochondrial DNA did support a split between *L. l. melanuroides* and *L. l. limosa*/*L. l. islandica* and also demonstrated two *L. l. limosa* haplotype clusters that were not geographically isolated. The genetic patterns probably resulted from a scenario of isolation of *L. l. melanuroides* from *L. l. limosa* in Beringia during the Last Glacial Maximum, possibly followed by recent introgression. During the Pleistocene separation of *L. l. islandica* from *L. l. limosa* occurred, followed by colonization of Iceland by the *L. l. islandica* during the Holocene. Founder events within *L. l. limosa*, followed by population expansion, took place during the Holocene also.

The aim of this thesis was to investigate long term population dynamic processes in Black-tailed Godwit populations resulting from habitat fragmentation on three different spatial scales by using genetics. We demonstrated that egg shell membranes provide a good alternative DNA source for population genetic studies compared to blood samples. Through the usage of egg shell membranes we could conduct the genetic studies in this thesis a large spatial scales. Genetic population structuring was studied here because Black-tailed Godwit breeding areas seemed geographically fragmented and it was unclear if this had led to genetic separation and inbreeding as well. On a local scale we did not detect any genetic populations structuring as an effect of habitat fragmentation. We showed that habitat fragmentation, although geographically visible, has not yet lead to genetic differences between Dutch breeding populations. As such it seems that Dutch breeding areas of the Black-tailed Godwit consist of a single panmictic population. On a global scale this thesis confirmed the presence of three genetically distinct groups now recognized as subspecies. However, no clear genetic differences were found between *L. l. limosa* across most of its current breeding range. Possibly, there is some genetic differentiation between *L. l. limosa* breeding areas in the Netherlands and Sweden.

Samenvatting

Toen hoogvenen werden omgevormd tot natte gras en hooilanden ten behoeve van de zuivelproductie, verschoof de opportunistische grutto van zijn vroegere broed habitat naar deze nieuwe gevormde cultuurlanden. In het Nederland van 1900 kwamen de grutto's vooral voor in deze vochtige hooilanden, die rond deze tijd tevens een groot deel van de Nederlandse grond besloegen. Door de gestage groei van mest gebruik op de hooilanden vanaf 1900 groeide de voedselbeschikbaarheid voor grutto's. Als een gevolg hiervan nam de grutto-broedpopulatie enorm toe in de eerste helft van de twintigste eeuw. Echter door steeds verdere landbouwintensivering, het oprukken van stedelijk gebied en de afname van het totale graslandareaal, is de habitat kwaliteit van geschikt gruttobroedgebied afgenomen met habitatfragmentatie als gevolg. Hierdoor neemt de kuikenoverleving waarschijnlijk al sinds eind jaren zestig af. Vanaf toen laten populatie aantallen een jaarlijkse afname zien van zo'n 5%.

Verschillende beheersstrategieën, zoals nestbescherming, het creëren van weidevogelreservaten, en mozaïekbeheer zijn ontwikkeld om de afname van de grutto en andere weidevogels te stoppen door het verbeteren van het nestsucces en habitatkwaliteit. Echter tot nu toe blijft het onduidelijk of deze beheersstrategieën uiteindelijk het gewenste effect hebben op habitatkwaliteit en dus habitatfragmentatie. Daarnaast is het momenteel nog steeds onduidelijk of habitatfragmentatie überhaupt een groot effect heeft op gruttopopulaties.

Habitatfragmentatie en veranderingen in habitatkwaliteit kunnen een effect hebben op de populatiedynamiek van een soort. Soorten kunnen bijvoorbeeld als gevolg van afnemende habitatkwaliteit een ander verspreidingspatroon laten zien of kunnen geïsoleerd raken van elkaar door habitatfragmentatie. Dit soort populatiedynamische veranderingen kunnen weer effect hebben op het genetische patroon van de soort. In dit proefschrift is genetica gebruikt om populatiedynamische processen te onderzoeken als gevolg van habitatfragmentatie op drie schaal niveaus. Voordat er gestart kon worden met dit genetische onderzoek moesten er eerst genetische merkers ontwikkeld worden en werd de toepasbaarheid van eischaalmembranen als DNA bron getest.

In hoofdstuk 2 hebben we de ontwikkelde microsatellieten gebruikt om te testen of eischaalmembranen een goed alternatief waren voor bloed monsters in populatiegenetisch onderzoek. We laten statistisch zien dat genetische informatie in eischaalmembraan-DNA, in vergelijking met DNA uit bloed, niet extra aangetast is door degradatie of contaminatie. Daarnaast, bleek ook geen sprake van DNA-degradatie of contaminatie in complete genotyperingen met bloed van hetzelfde individu als referentiegenotype. Ons onderzoek laat duidelijk zien dat eischaal membranen gebruikt kunnen worden voor populatiegenetisch onderzoek.

In hoofdstuk 3 onderzochten we de bevindingen van een eerdere studie die liet zien dat intronische variatie in het CHD1-Z gen (Z*) in grutto's gecorreleerd was met habitatkwaliteit op een lokale schaal opnieuw. De vorige studie suggereerde dat grutto-broedpopulaties differentieel gestructureerd waren in habitat van verschillende kwaliteit. Dit als een gevolg van positieve selectie druk in gruttopopulaties die broeden in habitat van hoge kwaliteit. Wij laten zien de aanwezigheid van het Z* allel niet gecorreleerd was met habitatkwaliteit. Andere analyses met fitnesscorrelatieven laten zien dat er tendens kan zijn dat vrouwtjes grutto's met het Z* allel eerder beginnen met broeden dan vrouwtjes zonder het Z* allel. De aanwezigheid van het Z* allel correleerde ook met een betere

conditie en een hogere terugkomkans van de gruttokuikens. Interessant was ook de bevinding dat kuikens die waren geboren op kruidenrijk grasland, dat over het algemeen grutto-broedhabitat is van hoge kwaliteit, een hogere terugkomkans hadden dan kuikens die op intensief boerenland waren geboren. Dit laat het grote belang van kruidenrijk grasland zien voor broedende grutto's.

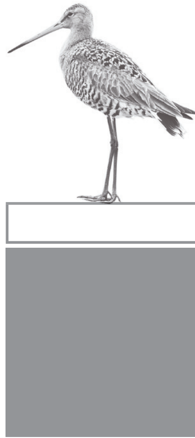
In hoofdstuk 4 en 5 onderzochten we of grutto-broedpopulaties in Nederland genetisch gestructureerd waren als gevolg van habitat fragmentatie. We demonstreren dat genetische diversiteit middelmatig was. Verder leken de Nederlandse grutto-broedpopulaties niet genetisch geclusterd. Genetisch uitwisseling tussen gemonsterde broedpopulaties was hoger dan '1 migrant per generatie'. Ook werd er geen isolatie-door-afstand-effect op de grutto-broedpopulaties gevonden. Deze resultaten toonden aan dat genetische diversiteit in de grutto-broedpopulaties werd onderhouden door voldoende genetische uitwisseling tussen die broedpopulaties. Nederlandse grutto-broedpopulaties lijken op genetisch vlak niet te zijn aangetast door habitatfragmentatie.

In hoofdstuk 6 hebben we ons onderzoek naar de effecten van habitatfragmentatie op grutto-broedpopulaties opgeschaald naar een nog groter schaal niveau. De genetische populatiestructuur en fylogenie van de grutto werd geanalyseerd waarbij broedpopulaties werden bemonsterd van IJsland tot het Baikal meer in Oost Rusland. Tevens werden hierin alle subsoorten meegenomen die momenteel bekend zijn binnen de grutto. Voor het gevonden genetische patroon dat voortkwam uit de mitochondrale en microsatellitenanalysen werd een historische verklaring gegeven. We demonstreren dat de genetische structuur binnen de *L. l. limosa* slechts zeer beperkt was volgens de microsatellitenanalyse. Daarnaast was er sprake van genetische differentiatie tussen de *L. l. limosa* en *L. l. islandica* maar niet tussen de *L. l. limosa* en *L. l. melanuroides*. De genetische patronen die de mitochondriale analyses lieten zien waren echter inconsistent met het patroon in het nuDNA. Het mitochondriale DNA liet een genetische split zien tussen *L. l. melanuroides* en *L. l. limosa/L. l. islandica*. Daarnaast vielen de *L. l. limosa* samples uiteen in 2 cluster die geografisch niet geïsoleerd van elkaar voorkwamen. Deze genetisch patronen zijn waarschijnlijk het resultaat van een scenario waarbij de *L. l. melanuroides* geïsoleerd raakte van de *L. l. limosa* in Beringia tijdens de laatste grote ijstijd, waarna mogelijk recent weer introgressie heeft plaats gevonden tussen deze subsoorten. Tijdens het pleistoceen zijn de *L. l. islandica* en *L. l. limosa* van elkaar gescheiden, gevolgd door kolonisatie van IJsland door de *L. l. islandica* tijdens het holoceen. Tijden het holoceen hebben foundermomenten binnen de *L. l. limosa*, gevolgd door populatie expansie op verschillende plekken, gezorgd voor het genetische patroon van deze gruttosubsoort.

Het doel van dit proefschrift was het onderzoeken van langdurige populatiedynamische processen in de grutto als resultaat van habitatfragmentatie op drie verschillende schaal niveaus door het gebruik van genetica. We laten zien dat eischaalmembranen, als DNA-bron in populatiegenetische studies, een goed alternatief zijn voor bloedmonsters. Door het gebruik van eischaalmembranen konden we het genetisch onderzoek in dit proefschrift uitvoeren op groot schaal niveau. Genetisch populatiestructuur werd hier bestudeerd omdat grutto-broedgebieden een geografisch gefragmenteerd lijken te zijn geraakt. Het was onduidelijk of dit ook tot genetische splitsing en inteelt had geleid tussen en binnen deze broedpopulaties. Op een lokale schaal konden we geen genetische populatiestructuur

waarnemen als resultaat van fragmentatie van habitat met een hoge kwaliteit. We laten zien dat habitatfragmentatie, hoewel geografisch zichtbaar, nog niet heeft geleid tot genetische verschillen tussen Nederlandse grutto-broedpopulaties. Het lijkt er op dat Nederlandse grutto-broedpopulaties gezien kunnen worden als één panmictische populatie. Op wereld schaalniveau laat dit proefschrift zien dat er op genetisch vlak drie subsoorten onderscheiden kunnen worden. Binnen het broedareaal van de *L. l. limosa* zijn echter geen duidelijke genetische verschillen gevonden. Wellicht is er sprake van lichte genetische differentiatie tussen *L. l. limosa* broed gebieden in Nederland en Zweden.

Photo: Jasper Doest



Curriculum Vitae

Krijn Baptist Trimbos was born in Leiden on the 6th ofOctober in the year 1979. After successfully finishing his VWO at the Rijnlands Lyceum in Oegstgeest he started studying biology at Leiden University in 1999. He finished his Propadeutics in 2000 after which he started a specialization track in medical biology and after that in environmental biology. During this time he conducted on three internships, two were done at the Leiden University Medical Centre (LUMC) and one was conducted at the Institute of Environmental Sciences Leiden (CML). His internships involved studying acquired systemic B cell memory in response to Human Papilloma Virus infection (2004 with Prof van den Burg), acquired systemic T cell memory in the Balinese population in response to Human Papilloma Virus infection (2005 with Prof. Lex Peters as a supervisor) and differences in breeding success of the Skylark at conventional and organic arable farms (2006 with Steven Kragten as a supervisor). His biology doctorate was finalized in 2007 after a literature study on the complexity of the human genome.

In 2007 he was research assistant for a short while at the Institute of Environmental Sciences Leiden (CML) focusing mainly on the value offield margins for Agricultural biodiversity in Zeeland, with special attention for the presence of butterflies and birds. At the end of 2007 Krijn started his PhD on genetic patterns of Black-tailed Godwit populations and their implications for conservation at the Institute of Environmental Sciences Leiden (CML) resulting in this thesis. During his PhD he supervised two students during their internships, gave several scientific lectures, assisted in a number of scientific courses and attended several symposia. Prof. de Snoo and dr. Musters were his supervisors during this time. Since 2011 he works at Landschap Noord Holland as a project manager meadow bird protection. The work there involves the coordination, training and education of volunteer meadow bird protectors and writing and executing research projects involving meadow bird conservation.

Publications

Trimbos JB, Mouw R, Ranke G, **Trimbos KB** and Zwinderman K (2002). *The Donati stitch revisited: favorable cosmetic results in randomized clinical trial*. Journal of Surgical Research, 107, 131-134.

Kragten S, **Trimbos KB** and de Snoo GR (2008). *Breeding Skylarks (Alauda arvensis) on organic and conventional arable farms in the Netherlands*. Agriculture, ecosystems and environment, 126, 163-167.

Verkuil YI, **Trimbos KB**, Haddrath O, Baker OJ and Piersma, T (2009). *Characterization of polymorphic microsatellite DNA markers in the black-tailed godwit (Limosa limosa: Aves)*. Molecular Ecology Resources, 9(5), 1415-1418.

Trimbos KB, Broekman J, Kentie R, Musters CJM and de Snoo G (2009). *Using eggshell membranes as a DNA source for population genetic research*. Journal of Ornitholog y, 150(4), 915-920.

Trimbos KB, Musters CJM, Verkuil Y, Kentie R, Piersma T and de Snoo GR (2011) *No evident spatial genetic structuring in the rapidly declining Dutch Black-tailed Godwit Limosa l. Limosa population*. Conservation Genetics, 12, 629-636.

Van der Velde M, Galama Y, Hooijmeijer J, **Trimbos K** and Piersma T (2011). Black-tailed Godwit Limosa limosaforensics: who laid a strangly shaped depredated egg? Limosa, 84, 155-161.

Trimbos KB and Muster CJM (2012). *Genetic differentiation between Dutch Black-tailed Godwit breeding sites re-examined using D statistics*. Wader study group bulletin, 119, 59-60.

Trimbos KB, Kentie R, van der Velde M, Hooijmeijer JCEW, Poley C, Musters CJM, de Snoo GR and Piersma T (2013). *Intronic variation on the CHD1-Z gene in Black-tailed Godwits Limosa limosa: correlations with fitness components revisited*. Ibis, 155, 508-517.

Kentie R, Hooijmeijer JCEW, **Trimbos KB**, Groen NM and Piersma T (2013). *Intensified Agricultural use of grasslands reduces growth and survival of precocial shorebird chicks*. Journal of Applied Ecology, 50, 243-251.

Trimbos KB, Doorenweerd C, Kraaijeveld K, Musters CJM, Groen NM, de Knijff P, Piersma T and de Snoo GR. *Patterns in nuclear and mitochondrial DNA reveal historical and recent isolation in the Black-tailed Godwit (Limosa limosa)*. Submitted PLOS One.