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Changed genetic variation in the vulnerable Swedish Corncrake (*Crex crex*) population: signs of immigration?

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Table of contents

Abstract

The Corncrake (*Crex crex*) is a globally declining species which has been severely affected by the intensification of agriculture. This study has investigated how the reduction in Corncrake numbers has affected the genetic variability in the Swedish population, by analysing samples from present individuals as well as from old museum specimens using species-specific microsatellite loci. The oldest temporal population, consisting of samples from 1863 to 1907, was genetically different compared to the following ones and had lower expected heterozygosity and allelic richness. During this time, early references has estimated the population to consist of approximately 60 000 pairs. Samples from 1863 to 1940 showed together signs of a recent reduction in numbers indicated by a heterozygosity excess, which would correlate in time with the great land conversion projects conducted in Sweden during the 19th century and the following mechanization of agricultural practices. Although Corncrake numbers decreased from 60 000 pairs at the end of the 19th century to less than 1000 ones in the 1950s, a BOTTLENECK analysis on the present population could not detect signs of this event. This is probably due to a recurrent immigration of birds with different genotypes, presumably from eastern European countries, which would have masked a possible lowered genetic variation in the native Swedish population. Even if this inflow occurred also during the time of the old samples' collection, it might have passed undetected due to the large population size, but when numbers of native Corncrakes later decreased it became apparent.

Keywords: *Crex crex*; genetic variation; microsatellites; museum samples

Sammanfattning

Kornknarren (*Crex crex*) är en globalt minskande art som har påverkats starkt av det intensifierade jordbruket. Denna studie har undersökt hur minskningen av antalet kornknarrar har påverkat den genetiska variationen hos den svenska populationen, genom att analysera prover från nutida individer samt från gamla museiexemplar med hjälp av artspecifika mikrosatteliter. Den äldsta tidsmässiga populationen, bestående av prover från 1863 till 1907, var i jämförelse med de följande genetiskt olika, samt hade lägre förväntad heterozygotigrad och antal alleler för sin provstorlek. Under denna tid bestod populationen, enligt tidiga referenser, av cirka 60 000 par. Prover från 1863 till 1940 visade tillsammans tecken på en tidigare nedgång i antal individer genom ett överskott av heterozygoter, vilket tidsmässigt bör sammanfalla med den stora svenska uppodlingen under 1800-talet och den efterföljande mekaniseringen av jordbruket. Trots att antalet kornknarrar minskade från 60 000 par i slutet av 1800-talet till mindre än 1000 på 1950-talet hittades inga spår av denna förändring i en BOTTLENECK-analys som gjordes. Detta beror förmodligen på en återkommande invandring av fåglar med andra genotyper, förmodligen härrörande från Östeuropa, vilket kan ha dolt en möjlig minskning av den genetiska variationen hos inhemska kornknarrar. Även om detta tillskott pågick även under den tid då de gamla proverna samlades in kan det ha passerat obemärkt på grund av den dåvarande stora populationsstorleken, men började senare, under den följande nedgången i antal inhemska kornknarrar, märkas.

Nyckelord: *Crex crex*; genetisk variation; mikrosatelliter; museiprover

Introduction

Destructive human activities are reducing the global diversity on earth at an accelerating rate, this is sometimes referred to as the sixth mass extinction (Höglund 2009), which may involve a loss of over 30 % of all living species (Myers 1987). Habitat loss and alteration today imposes the largest threat to over half of the worlds red-listed bird species, and within the next 100 years up to 1 200 of those are facing the risk of extinction (Crosby *et al*. 1994).

It has been argued that populations residing at the margin of their species range are especially important for the long-term survival of the species and maintenance of genetic variation (Hampe & Petit 2005), a factor which is necessary for populations and species' ability to respond to future environmental changes through natural selection. In small populations, the genetic variation is lost faster than in large due to accelerated importance of stochastic processes such as genetic drift, which results in non-adaptive changes in allele frequencies over generations and a risk of accumulating deleterious mutations (Lande 1988; Allendorf & Luikart 2007). This means a reduction in fitness at both the individual and population level, which may eventually lead to lowered reproductive rates as well as increased mortality and entrap the small population in a negative spiral known as the extinction vortex (Fagan and Holmes 2006). Changes in environmental conditions often work synergistically with genetic factors, and enhance the negative effects of low genetic variability of a species or population. Inbred populations have also shown to suffer more from negative environmental changes than non-inbred ones. For many small and endangered populations, environmental risks induced by humans may even be the greatest threat and affect the populations before the genetic problems become apparent (Lande 1988; Bijlsma *et al*. 2000).

Many extinction events of taxa are however often preceded by great losses of genetic diversity which makes it possible to foresee these events using genetic approaches. For example, the limited gene flow between fragmented populations results in increased frequencies of inbreeding and therefore decreases in average heterozygosity, H_e (Höglund 2009), a measure that has been found to be 35 % lower in threatened taxa than in non-threatened (Spielman *et al*. 2004). This is a good general measure of genetic variation within a population and has the advantage of being very insensitive to sample size. Good estimates can therefore be made with only a few individuals, as long as the analysis involves many loci (Nei 1978). Another measure is the allelic richness, which is the number of alleles per locus measured by the number of sampled genes (Allendorf & Luikart 2007). While heterozygosity is a good indicator of a populations' ability to adapt through natural selection directly after a great reduction in numbers, the allelic richness is more sensitive in actually detecting these decreases and is important for the long term response to selection and therefore for the survival of the population (Allendorf 1986).

A type of molecular marker that is commonly used in population genetics is microsatellites, which consists of short DNA sequences of one to six base pairs that are repeated approximately five to 100 times (Allendorf & Luikart 2007). The small size of microsatellite loci allows successful amplifications of old and degraded DNA (Bruford & Wayne 1993) and they also have faster mutational rate than other parts of the genome, in birds reported as high as 2.7 – 7.1 % per meiosis (Primmer *et al*. 1998), making them highly polymorphic and thus very suitable for studies of endangered populations with few individuals (Allendorf & Luikart 2007). This rapid mutation results in changes in allele frequencies over relatively short time periods which allow detection of recent evolutionary events such as severe reductions in population size known as bottlenecks (Freeland 2005). These are important to identify since affected populations suffer from an increased extinction risk and therefore may be in need of conservation actions, and for that reason the computer program BOTTLENECK (Cournet &

Luikart 1996) has been developed to detect previous population declines in contemporary samples. The program tests for a "heterozygosity excess" in the population and requires at least 10 microsatellite loci and samples from at least 30 individuals to achieve proper statistical power (Luikart & Cournet 1998; Piry *et al.* 1999). This "heterozygosity excess" generally distinguishes bottlenecked populations, and is defined as a greater population heterozygosity, H_e, than would be expected if the population were in equilibrium between genetic drift and mutation, H_{eq} (calculated from the number of observed alleles at the locus of interest and the number of sampled individuals), i.e. $H_e > H_{eq}$ (Cournet & Luikart 1996). The excess is caused by the fact that rare alleles are rapidly lost due to accelerated genetic drift during a population reduction (and thus reducing the expected equilibrium heterozygosity, H_{eq}), in contrast to the relatively bottleneck-insensitive measure of heterozygosity (H_e). This evidence of a population bottleneck is however temporary and will only be detectable until a new mutation-drift equilibrium has been established, which occurs when the number of generations after the reduction is equivalent to approximately 0.2 to 4 times the populations' effective size (N_e) (Luikart & Cournet 1998). The computer program is however not always able to detect previous population decreases, which may happen when new mutation-drift equilibrium is rapidly set due to small effective population size and short generation time. Pre-bottlenecked data such as old museum specimens can therefore provide important information about the historical genetic variation within a population, and may reveal losses of genetic variation not detectable when only analysing contemporary samples (Larsson *et al.* 2008).

Study species

The Corncrake is a medium-sized Rallidae species who lives a secretive life hidden in tall vegetation. It prefers drier areas than other rail species and its original breeding habitat is thought to have been coastal and riverine meadows and grasslands in alpine and fire-created areas dominated by Carex, Iris and Typhoides species (Green *et al.* 1997). The key factor determining suitable breeding areas is the vegetation height, which has to be at least 20 cm high to properly conceal the birds but not so dense that it prevents them to easily walk through it (Tyler 1996). With the modern intensified agriculture these original habitats have become scarce and the species is now found breeding in secondary habitats, mainly in grasslands used for hay and silage production but also in crop fields, clear-cut forests, pastures and abandoned land (Green *et al.* 1997).

In March and April Corncrakes migrate from their wintering areas in the savannas of south-central and south-east Africa, arriving to the breeding areas in April-May. This breeding range has historically been extending over large parts of Europe between 41°N and 65°N and as far as 120°E in central Asia (Crockford *et al.* 1996). Nowadays the distribution (Figure 1) has been restricted and in western and central Europe the population is fragmented (Green *et al.* 1997).

In Europe, the number of Corncrakes has been declining during the last 150 years (Gallo-Orsi 2001) and over the recent decades the rate has accelerated to an average of 20-50 % loss. This decline has been most severe in Western Europe, and the species was on the brink of extinction in some countries. However, in the last ten years it seems that this trend has been broken and Corncrake numbers are again increasing in several countries. The global number of territorial males is now estimated to 1.7 to 3.5 million (Koffijberg & Schäffer 2006), with more than half of the population breeding in Asia (Russia, Georgia, Iran, Afghanistan, Tajikistan, Kyrgyzstan, Kazakhstan and China). In Europe, the species is breeding in 34 countries, with a total number of 92,000–233,000 males. A large part of those are found in the central and eastern parts (such as Belarus, Russia, Ukraine and Bulgaria) and France is the only western state holding more than 1,000 males (Crockford *et al.* 1996).

In the 18th and 19th centuries, the Corncrake was a common and well-known species in the Swedish agricultural landscape (Ericson & Tyrberg 2004) with estimates of approximately 60,000 pairs at the end of the 19th century (Svensson *et al.* 1999). Then, however, the species had already begun declining, and between 1910 and 1940 the most sever reduction in numbers took place (Pettersson 2007). According to some sources, the species had a fragmented distribution in large parts of its national breeding range already by the 1920s (Jägerskiöld & Kolthoff 1926), and the low number of today is thought to have been reached in the late 1940s (Risberg 1988). Since the 1950s, the decline decelerated and in the last decades of the 20th century Corncrake numbers were increasing. Large yearly fluctuations have characterized the population during the latter half of the 20th century (Bengtsson 2005), and the current number of territorial males is estimated to vary around 800 (Pettersson 2007). Three major breeding areas are recognized in Sweden; the provinces of Uppland and Västmanland in the central-eastern part of the mainland and the islands Öland and Gotland in the Baltic Sea (Figure 1). Whereas the mainland area has shown a recent increase in the number of calling territorial males, a decrease has occurred in the latter two, but overall the national population trend now seem to be stable (Ottvall 1999).

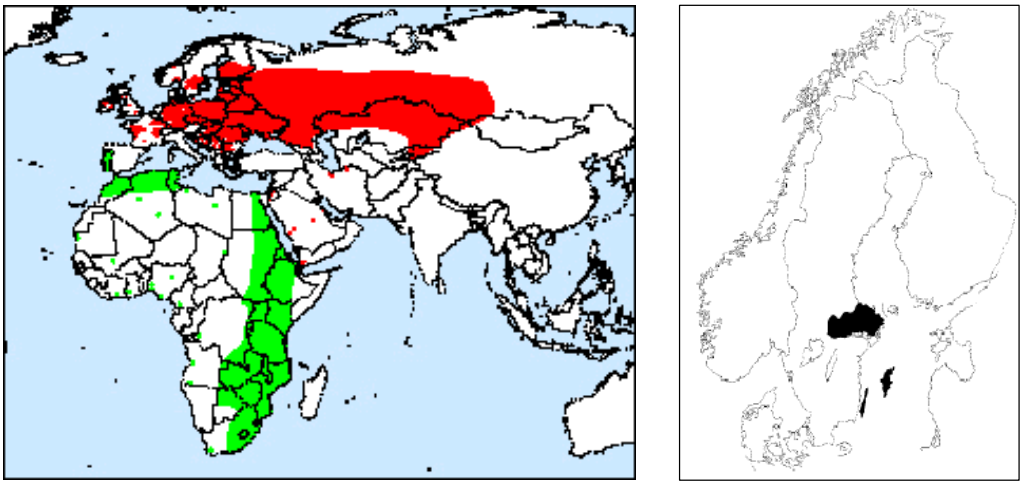


Figure 1 The present global distribution of Corncrakes, with wintering areas coloured green and breeding areas red. Important Swedish breeding areas shown to the right. From BirdLife International (2008) and Ottvall (1999) respectively.

The main reason for the species' global decline is the intensification of agricultural practices with replacement of manual mowing by mechanization (Green *et al.* 1997) and the conversion of many meadows to croplands with subsequent drainage (Risberg 1987). Between 1700 and 1870, the agrarian revolution took place in Sweden with increases of arable land with about 350 % on behalf of meadows and pastures. From the 1820s and onwards, large parts of the Swedish wetlands were drained in national, state subsidised, projects in order to create new croplands and improve existing ones (Gadd 2000), leading to great losses of suitable Corncrake habitats. With the latter mechanization of agriculture in the 20th century, whole fields of silage and hay could be completely harvested earlier than was previously possible by hand, and in many European countries that mowing period has come to take place during or right after the birds hatching period in late June. Although adult and juvenile birds are fast enough to escape the machines, flightless chicks are often killed and nests with un-hatched eggs destroyed. Corncrakes can under favourable conditions produce a second brood, and since the adult survival rate is estimated to as low as 20-30 % this is thought to be required to sustain stable population numbers (Green 1999). Earlier mowing dates lead however also to habitat destruction, and there is little chance that vegetation in mowed fields will grow high

enough again to allow production of a second brood. Additionally, the fields with high growth rate are often mowed a second time, resulting in the destruction of also the eventual replacement nests (Green *et al*. 1997). This has made the species more or less restricted to countries with low production rates and old-fashioned technology, such as the former Soviet-union dominated countries (Gallo-Orsi 2001). Although the agriculture became partly mechanized during the Soviet era, an absence of incitement for workers to optimize the production resulted in remote and difficult land areas being left undisturbed, providing good breeding success for Corncrakes and distinctively larger populations than in western countries. Since the collapse of the Soviet Union and the following economical crisis in several of the former eastern bloc countries, large agricultural areas have been abandoned due to privatisation and lack of fuel, allowing even greater population increases (Schäffer & Green 2001). This has triggered population numbers also in western states, and in Sweden the increase in the last decade is mainly thought to result from an influx of birds from the large populations in the east European region (Berg *et al.* 2004; Bengtsson 2005; Pettersson 2007). However, these favourable breeding conditions are only temporary since natural succession in the abandoned areas will result in accumulated coverage of bushes and trees, thus eventually preventing Corncrake breeding. It is also likely that political reforms will take place in these countries, with development in agricultural practices and intensification comparable to Western Europe, which in the future may lead to similar negative population trends (Schäffer & Green 2001).

The species' history of extensive population declines has made it classified as "Near Threatened" on the global scale (BirdLife International 2006), and at the European level it is placed in the highest threat category (SPEC 1) in the Species of European Conservation Concern (Burfield & van Bommel 2004). It is included in Annex I of the European Union Birds Directive (1979), in Appendix II of the Bern Convention (1979) and in Appendix II of the Bonn convention (1997). In Sweden, Corncrakes are considered as "Vulnerable" (Gärdenfors 2005). At the global and the European scale, two action plans have been developed to promote and coordinate conservation actions (see Koffijberg & Schäffer 2006 and Crockford *et al*. 1996 respectively) and in Sweden, as well as in other countries, a national plan has been developed (Pettersson 2007).

Information is scarce on whether gene flow takes place between the fragmented European populations, and the possibility exists that they are divided by barriers into several small subpopulations (Wettstein & Schäffer). A study of the return rate of Corncrakes to their birthplace has however showed signs of strong yearly immigration, and genetic analyses has revealed very weak differentiation between European populations (F_{ST} = 0.004). When grouping populations into eastern, western and northern regions, only the former two showed a significant differentiation between each other (Wettstein 2002).

Aim of the study

This study is testing the hypotheses that the Corncrake population in Sweden has lost genetic variation during the habitat destruction and mechanization of agriculture in the early 1900s and that the great reduction in numbers during that time had the effect of a population bottleneck. This would be important to know since a "history of inbreeding" follows a population that has undergone a bottleneck which, if stressful environmental changes occur, will lend it an increased risk of extinction although it has recovered in numbers (Bijlsma *et al*. 2000). The hypothesis is examined by testing for heterozygosity excess in the contemporary population as well as comparing allele frequencies in old museum specimens and contemporary individuals, using species-specific microsatellite loci.

Materials and methods

Data collection

Samples were taken from 22 stuffed Corncrakes (19 toe pads and three tissue samples in ethanol,) stored at the Swedish Museum of Natural History and collected throughout Sweden between 1863 and 1996 (see Appendix 1). As a comparison with the historical museum samples I used eight blood samples collected from captured birds during the summer 2008 at Skåne in the south of Sweden. Blood samples from two birds and DNA from three birds collected in 2003 at Öland and 2001 at Gotland respectively, both in the east of Sweden, were also used as contemporary references.

For the museum samples, genomic DNA was extracted from ~ 1 * 1 mm^2 tissue using Qiagen DNeasy Blood and Tissue kit with the protocol Isolation of Total DNA from Animal Tissues. The manufacturer's instructions were followed, apart from the following modifications. To pulverize the toe pads, an initial step was added in which the samples were grinded in liquid nitrogen together with steal beads. 20 µl 1 M DTT was added before the over night incubation to completely lyse the samples. After this step, 300 µl manufacturer's buffer AL was added, followed by 1 µl of carrier RNA. After a 10 minute incubation (70 ºC), 300 µl 96 % ethanol was added. To increase the final DNA concentration, elution was made with 100 and 50 µl of manufacturer's buffer AE. For the blood samples, the DNA was extracted using a salt purification, in which 340 µl SET-buffer, 12.5 µl proteinase K (10 mg/ml) and 20 µl 20 % SDS were used to lyse the cells during an over night incubation (50 ºC). After that, 300 µl NaCl was added followed by 20 seconds vortexing and a 10 minute centrifugation (13 000 rpm), then 150 µl Tris (0.01 M pH 8.0) and 750 µl cold 2-propanol was added to a new tube containing 600 µl of the supernatant, which was left in the freezer for $\geq$ 30 minutes for the DNA to precipitate. The samples were then centrifuged in 15 minutes at 10 700 rpm after which the supernatant was thrown away and the pellet washed with 1 ml cold 70 % ethanol, followed by a last centrifugation for 10 minutes (10 700 rpm). After an over night drying the pellet was dissolved in 100 µl TE buffer (pH 7.6). The DNA samples were diluted ten times with ddH_20 before further use.

Extracted DNA was amplified with polymerase chain reaction (PCR), using fluorescently labelled primers for nine Corncrake microsatellite loci (Crex1, Crex2, Crex4, Crex6, Crex7, Crex8, Crex9, Crex11 and Crex12) developed by Gautschi *et al*. (2002). The first primer pair (Crex1) was however later excluded due to low functionality, and it had also previously been found to deviate significantly from Hardy-Weinberg proportions indicating presence of null alleles (Gautschi *et al*. 2002). Amplifications were carried out in 10 µl mixtures prepared in a separate pre-PCR room, containing 5 µl multiplex mastermix (Qiagen), 2 µl RNA:se free water (Qiagen), 10 µl of each forward and reverse primer and 2 µl DNA. The same thermal profiles as described by Gautschi *et al*. (2002) was used on a ABI GeneAmp® PCR System 2700, a Eppendorf Mastercycler® gradient and a VWR UnoCycler Thermal Cycler simultaneously. The annealing temperatures (T_a) used were 55 ºC for Crex2, and 57 ºC for Crex4, Crex6, Crex7, Crex8, Crex9, Crex11 and Crex12. For the latter two loci, the annealing temperature was later changed to 58 ºC, which was the temperature found to best suit the loci. Since amplification errors is a recognized problem when using museum samples (Sefc *et al*. 2003), at least three PCR sets were carried out for each sample and locus.

Data analysis

The amplified products were diluted and mixed with MegaBACE Size Standard, and the fragments analysed on a MegaBACE 1000 DNA Analysis System using the software MegaBACE Genetic Profiler Version 1.2 (©Amersham Biosciences 2003) to detect the alleles. To obtain reliable genotypes, an allele had to appear at least twice to be accepted (Miller and Waits 2003). The samples were divided into populations based on time period, with samples collected between 1863 and 1907 classified as Old, between 1919 and 1940 as Semi-old, between 1946 and 1968 as Semi-present and those between 1996 and 2008 as Present. "Old", represents the population when it was still large, "Semi-old" when the large decrease took place and "Semi-present" just after it had reached the size of today. "Present" should represent the contemporary population.

The observed heterozygosity (H_o), expected heterozygosity (H_e) and number of alleles were calculated with the Excel Microsatellite Toolkit 3.1 (Park 2001) in order to examine the genetic variation within and between each population. The computer program Fstat (Goudet 2001) was used to investigate the possibility of non-random mating within each population indicated as a deficit of heterozygotes (F_{IS}), which may indicate inbreeding or a hidden structure between the samples. The same program was also used to test for possible genetic divergence (F_{ST}) between the different populations, as well as the allelic richness, which corrects the number of alleles for sample size, in each population. These comparisons were made within and between all of the four populations (Old vs. Semi-old vs. Semi-present vs. Present), but also for the Present population versus the Old and Semi-old population together, resulting in one group of four populations and one group of two.

As a complement to the F_{ST} value, the genetic distances between all individuals in the four populations were analysed in the computer program GENALEX (Peakall & Smouse 2006), by conducting a principle coordinate analysis (PCA) on a distance matrix with standardized data.

To test if the documented decline in Corncrake numbers in the early 1900 led to declines in the genetic variation that can be detectable in the Present population, the program BOTTLENECK was run. It was also run on the Old & Semi-old populations (i.e. both tested together as one population) to check for possible changes in Corncrake numbers before the known large decline. Heterozygosity excess was in both runs tested under all three mutation models available; the infinite alleles model (IAM), stepwise mutation model (SMM) and two-faced model (TPM) of mutation. According to Piry *et al.* (1999), the probability of SMM in the latter model was set to 95 % and the variation to 12.

Results

The comparisons of genetic diversity showed that the Old population differed significantly to the following Semi-old population in expected heterozygosity (paired t-test: $P < 0.04$, d.f. = 7, t = 2.36) and allelic richness (paired t-test: $P < 0.04$, d.f. = 7, t = 2.36). There was also a sign of a difference in expected heterozygosity, based on SD (Table 1) between the Semi-old and the Semi-present population but the low sample size of the latter prevented significant t-test results. All populations, except the Semi-present, showed significant deficits of heterozygotes by positive F_{IS} values ($P < 0.004$ for group one; $P < 0.002$ for group four). No differences were found in observed heterozygosity (H_o) between any of the populations, neither were there any signs of genetic divergence between them based on the pairwise F_{ST} values (Table 2). However, the scatterplot (Figure 2) of genetic distances between all individuals from the principle coordinate analysis (PCA) showed that all but one of the individuals from the Old population was, together with two individuals from the Semi-old population, aggregated in a cluster away from the other individuals. The two axes in the plot explained up to 47 % of the variance in the data.

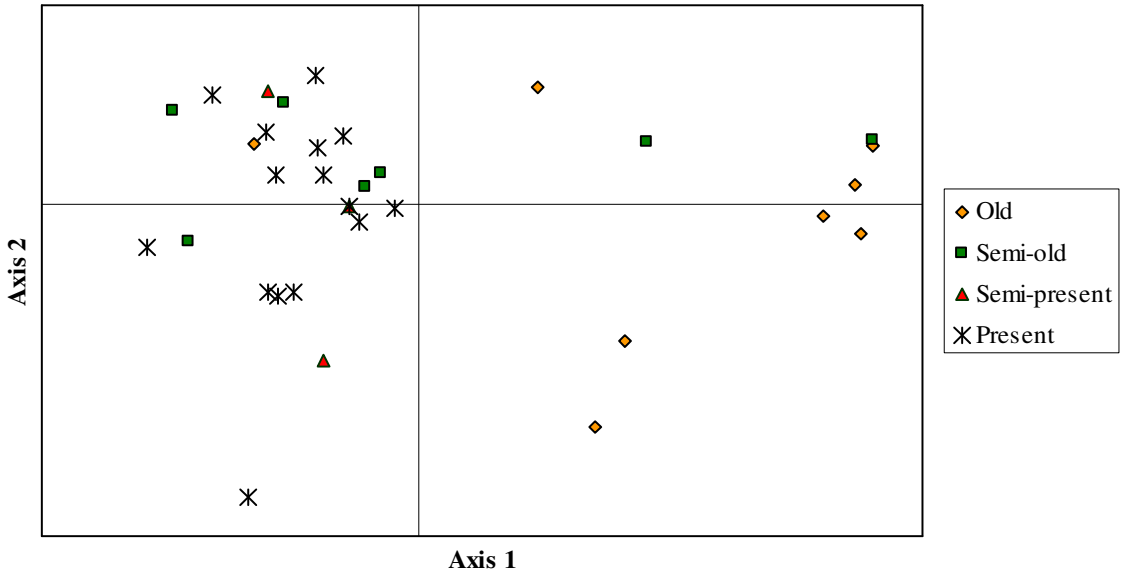


Figure 2 Genetic distances between all individuals in the four populations, based on a principle coordinate analysis (PCA) of a distance matrix with standardized data. Axis 1 and 2 explains together up to 47 % of the variation.

Table 1 Measures of genetic diversity for four and two populations respectively. Allelic richness calculations for the first group are based on a minimum sample size of two diploid individuals, and for the latter on eight diploid individuals. Significant F_{IS} are indicated by * (for group one based on 640 randomisations with indicative adjusted nominal level (5%) for one table: 0.00156 and for group two based on: 320 randomisations with indicative adjusted nominal level (5%) for one table: 0.00313).

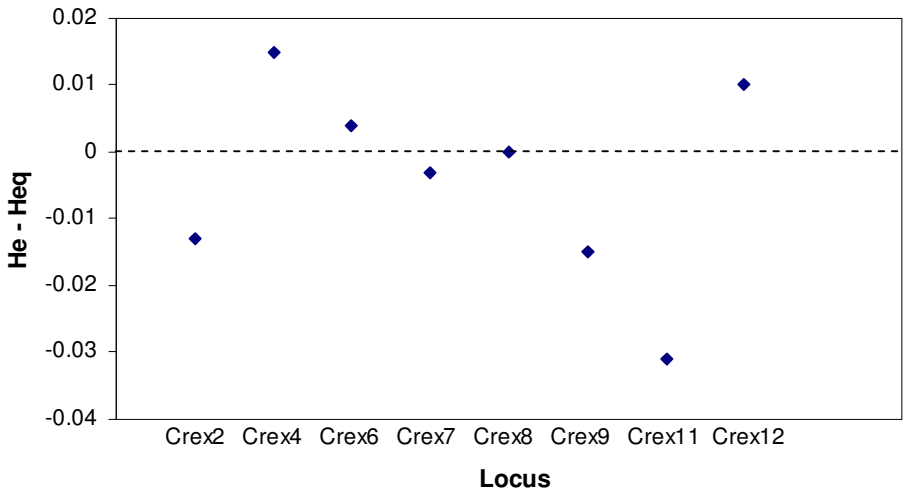
Population	Sample size	Sample year	H_e	H_e SD	H_o	H_o SD	No. of alleles	No. of alleles SD	Allelic richness	F_{IS}
Old	8	1863-1907	0.8989	0.0154	0.7336	0.0619	7.75	3.24	3.42	0.221*
Semi-old	7	1919-1940	0.9437	0.0120	0.8042	0.0556	9.25	1.67	3.68	0.160*
Semi-present	3	1946-1968	0.9083	0.0175	0.8333	0.0761	4.63	0.74	3.45	0.101
Present	15	1996-2008	0.9256	0.0134	0.8000	0.0365	15.00	3.07	3.59	0.140*
Old & Semi-old	15	1863-1940	0.9356	0.0051	0.7868	0.0406	13.1	3.09	10.31	0.166*
Present	15	1996-2008	0.9256	0.0134	0.8000	0.0365	15.0	3.07	10.42	0.140*

Table 2 Pairwise F_{ST} values for both four and two populations. P-values for the first group was obtained after 120 permutations with an indicative adjusted nominal level (5%) of 0.008333, and for the second after 20 permutations with an indicative adjusted nominal level (5%) of 0.050000. NS = non-significant difference.

	Old	Semi-old	Semi-present	Old & Semi-old
Semi-old	0.0047 (NS)			
Semi-present	-0.0303 (NS)	-0.0308 (NS)		
Present	0.0006 (NS)	-0.0066 (NS)	-0.0222 (NS)	-0.0066 (NS)

The program BOTTLENECK did not find any significant heterozygosity excess ($H_e > H_{eq}$) in the Present Corncrake population (Figure 2) that would indicate a recent bottleneck. Neither did the test for mode shifts in that population, which showed that allele frequencies had the normal L-shaped distribution found in stable populations. However, when running the program on the Old & Semi-old populations together (with samples collected 1863 - 1940), the results from the Wilcoxon test (Figure 2) showed a significant heterozygosity excess under all mutational models (IAM: $P < 0.01$; TPM: $P < 0.02$; SMM: $P < 0.02$). There were however no signs of mode shifts and the Old & Semi-old population was, just like the Present, classified as stable.

a)



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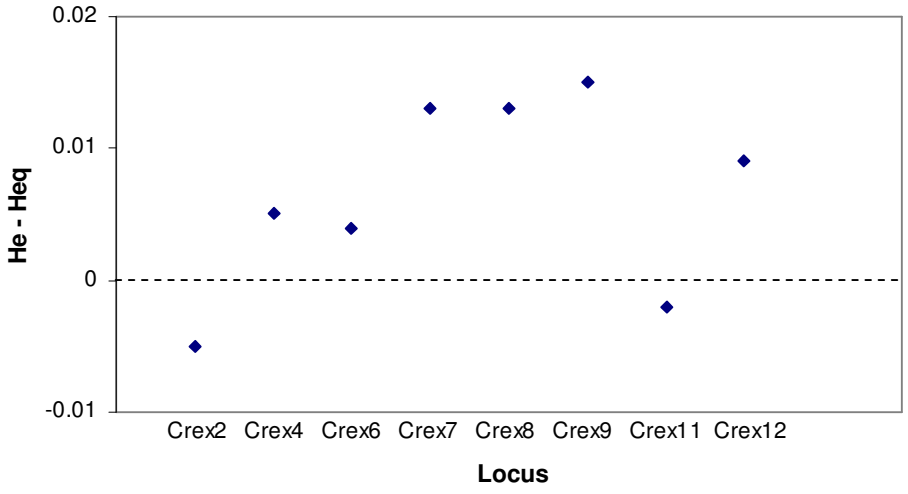

Figure 2 Relation between H_e and H_{eq} at each of the eight microsatellite loci in the Present (a) and Old & Semi-old (b) population, using the two-phased model (TPM) of mutation. The dashed line represents an equilibrium state, while points with positive or negative values represent loci with heterozygosity excess or deficiency respectively.

Discussion

The measure of expected heterozygosity (H_e) and allelic richness showed that the Old population had significantly lower H_e and fewer alleles for its sample size than the following Semi-old population. The principle coordinate analysis (PCA) indicated also that the formers genetic characters in general were different from those of the other populations' and especially from the two most recent ones. The measure of genetic differentiation (F_{ST}) between the populations was, nonetheless, not significant for any of the pairwise comparisons. It can however not be determined whether this is an accurate result, since there where too many missing alleles (due to amplification problems) in the Old population to enable P-values for the comparisons between this and the other populations. It can be mentioned though, that when excluding the deviating sample (originating from the latter part of the Old population which in the PCA plot was clustered together with individuals from the three more recent populations) from the calculation the F_{ST} value became higher between the Old population and the latter ones. It was still so low though, and without P-value, that no particular differentiation could say to be existing. Another aspect to this is that the contemporary European populations have been shown to be very weekly differentiated, $F_{ST} = 0.004$ (Wettstein 2002), and thus it would be likely to find the same pattern in Swedish populations, although these are separated by time rather then distance.

Due to low sample size and many missing alleles (because of amplification problems) in the Old population, the BOTTLENECK analysis could, unfortunately, not be performed on that population solely, but had to include also the Semi-old population. However, when running the program on those two populations together (with individuals from 1863 to 1907), evidence of recent losses of genetic variation was found. This suggested decline would have coincided in time with the large Swedish land reclamation and drainage projects during the 18th and 19th century that destroyed substantial areas of wetlands, meadows and pastures (Gadd 2000). Early documentations indicates that the Swedish Corncrake population actually was decreasing in the end of the 19th century (Pettersson 2007), but before that there is no census numbers that can help to establish whether the large decline that is indicated by BOTTLENECK has actually took place. It can, however, not be ruled out when considering the large destruction of suitable Corncrake habitat.

Based on the documented large decline in Corncrake numbers during the beginning of the 20th century, I expected to find signs of decreased variability in the present population compared to older ones. However, no sign of heterozygosity excess was found that would indicate a recent bottleneck. An explanation could be that immigration of birds with different genotypes than the "native Swedish" have concealed the effects of a previous bottleneck, which therefore remains undetected (Luikart & Cournet 1998). Based on the species' low survival rate it is believed that two broods each season are required to sustain a stable population (Risberg 1987; Green 1999), but with the absence of tall vegetation in the mid- and latter part of the breeding season due to earlier mowing dates, that is unlikely to happen very frequently and the Swedish population in general is not thought to be self-reproducing (Pettersson 2007). In fact, in the past decades there are few reports of verified breedings at all in the Swedish mainland (Risberg 1987). The number of calling males in Sweden has, despite this, been stable (but with strong yearly fluctuations) during the last decades, which is thought to result from immigration from other countries (Risberg 1987; Berg *et al*. 2004; Bengtsson 2005; Pettersson 2007). With this seemingly recurrent influx of birds from foreign populations, considered likely already in 1968 (Bengtsson 2005), the probability is not high that the program BOTTLENECK would find signs of a past population decrease (although it was severe) in the present population. Additionally, it should also be kept in mind that, when

analysing species with relatively short generation times, the program can fail to detect population decreases due to the hastened rate at which new mutation-drift equilibriums are set (Larsson *et al*. 2008). The relatively long time elapsed since the reduction of Corncrakes numbers could therefore be a supplementary explanation to the failure of the program in this study. It is also important to note that it is recommended that at least 10 loci and 30 individuals are included in a BOTTLENECK analysis (Luikart & Cournet 1998; Piry *et al*. 1999), wherefore both the results for the Old & Semi-old and the Present population, which neither fulfil the recommendations, should be interpreted with caution.

The F_{IS} value shows how the observed frequency of heterozygotes in a population deviates from that expected if random mating occurred. A positive F_{IS} means deficits of heterozygotes, and in this study all populations but the Semi-present was found to have significantly positive F_{IS} values. This means that mating within each population can not be considered as random which, theoretically, can be due to inbreeding within the populations. It will however also occur when two or more distinct subpopulations are unknowingly analysed as one large population (resulting in a so called Wahlund effect). Since the samples in this study are collected over a wide time span (1863 - 1907, 1919 – 1940 and 1996 – 2008 for the Old, Semi-old and Present populations respectively), it is likely that the deficit of heterozygotes is actually caused by the inclusion of many generations in each population, which means that all individuals can not be considered to have equal probability of mating with another randomly chosen individual. Strong yearly immigration has been shown to occur in European Corncrake populations (Wettstein 2002), and if this process takes place in Sweden it could also, together with a presumable Wahlund effect, have affected the positive F_{IS} value.

When summarising the results it appears as Swedish Corncrakes were decreasing in numbers already in the mid-1800s, although it was still large compared to what was to come, most likely due to land conversion. In the beginning of the 20th century something happened and from then on the populations seem to be genetically different from the Older. It was during this period that the larges decreases took place (Pettersson 2007) and it is possible that immigration, which might also have been a recurrent process earlier but then was concealed due to a dilution effect, all of a sudden became visible because of the low numbers of individuals left and altered the genetic make up of the Swedish population. The new genotypes would have masked the possible impacts the size reduction might have had on the genetic variation of native Corncrakes, and might explain why the BOTTLENECK analysis did not find sings of this event in the contemporary population.

Based on the findings in this study, there appears to be no current threat to the survival of Corncrakes in Sweden due to genetic impoverishment. Direct human activities seem to compose the most alarming danger to the population, wherefore conservation actions should focus on habitat protection and restoration, as well as implementing Corncrake friendly mowing methods. However, if the positive Corncrake trends in the former Soviet-union countries will reverse, because of political reforms, globally increasing food prices etc., then the future of the Swedish population will be more insecure and actions might have to be taken to ensure the long term survival of the population. It is not determined yet, though, whether the situation in Sweden originates from immigration of east European birds (or from immigration at all), wherefore it should be interesting to compare genotypes from Swedish Corncrakes with those from other populations across the Baltic Sea, as well as from the rest of Europe, to be able to assign eventual migrates to their “home population”.

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References

Allendorf, F. W. 1986. Genetic drift and the loss of alleles versus heterozygosity. *Zoo Biology* **5**: 181-190

Allendorf, F. W. & Luikart, G. 2007. *Conservation and the Genetics of Populations.* 1st edition. Blackwell Publishing.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. & Bonhomme, F. 1996–2004. GENETIX 4.05. logiciel sous Windows TM pour la ge´ne´tique des populations. Laboratoire Ge´nome, Populations, Interactions, CNRS UMR 5000. Universite´ de Montpellier II. Montpellier (France).

Bellinger. M. R., Johnson. J. A., Toepfer, J. & Dunn, P. 2003. Loss of Genetic Variation in Greater Prairie Chickens Following a Population Bottleneck in Wisconsin. U.S.A. *Conservation Biology* **17**: 717–724

Bengtsson, D. (Compiler). 2005. Riksinventeringsarter i SOF's regi. Swedish Ornithological Society's research committe. Available from http://www.sofnet.org/apps/file.asp?Path=2&ID= 2009&File=Riksinventeringsarter.pdf

Berg, Å., Gustafson, T. & Smedberg, A. 2004. Kornknarr - en problemart som "faller mellan stolarna"? In: Pehrson, I. & Svensson, R. (eds) *Hagmarks Mistra Årsrapport 2003*. Uppsala.

Bijlsma, R., Bundgaard, J. & Boerema, A. C. 2000. Does inbreeding affect the extinction risk of small populations?: predictions from Drosophila. *Journal of Evolutionary Biology* **13**: 502-514

BirdLife International. 2006. *Crex crex*. In: IUCN 2007. *2007 IUCN Red List of Threatened Species.* <www.iucnredlist.org>. Downloaded on 29 August 2008.

Bruford, M. W. & Wayne, R. K. 1993. Microsatellites and their application to population genetic studies. *Current Opinion in Genetics and Development* **3**: 939-943

Burfield, I. & van Bommel, F. 2004. Birds in Europe: population estimates, trends and conservation status. BirdLife International.

Cournet, J. M. & Luikart, G. 1996. Description and Power Analysis of Two Tests for Detecting Recent Population Bottlenecks From Allele Frequency Data. *Genetics* **144**: 2001-2014

Crockford, N., Green, R., Rocamora, G., Schäffer, N., Stowe, T. & Williams, G. (Compilers). 1996. *Action plan for the Cornkrake (*Crex crex*) in Europe*. Pre-publication version.

Crosby, M. J., Stattersfield, A. J., Collar, N. J. & Bibby, C. J. 1994. Predicting avian extinction rates. *Biodiversity Letters* **2**: 182-185

England, P. R., & Osler, G. H. R. 2001. GENELOSS : a computer program for simulating the effects of population bottlenecks on genetic diversity. *Molecular Ecology Notes* **1**: 111-113

Ericson, P. G. P. & Tyrberg, T. 2004. *The early history of the Swedish avifauna. A review of the subfossil record and early written sources.* Kungl. Vitterhets Historie och Antikvitets Akademiens Handlingar, Antikvariska serien 45. Stockholm.

Fagan, W. F. & Holmes, E. E. 2006. Quantifying the extinction vortex. *Ecology Letters* **9**: 51-60.

Freeland, J. R. 2005. *Molecular Ecology*. John Wiley & Sons Ltd. Chichester

Gadd, C-J. 2000. Den agrara revolutionen. In: Myrdal, J. (ed) *Det svenska jordbrukets historia*. Natur och Kultur/LTs förlag. Borås.

Gallo-Orsi, U. (ed) 2001. *Saving Europe's most threatened birds: progress in implementing European Species Action Plans*. BirdLife International. Wageningen.

Gautschi, B., Klug Arter, M., Husi, R., Wettstein, W. & Schmid, B. 2002. Isolation and characterization of microsatellite loci in the globally endangered Corncrake. *Crex crex* Linné. *Conservation Genetics* **3**: 451-453.

Goudet, J. 2001. FSTAT. a program to estimate and test gene diversities and fixation indices (version 2.9.3). Updated from Goudet (1995)

Green, R. E. 1999. Survival and dispersal of male Corncrakes *Crex crex* in a threatened population. *Bird Study* **46** (Suppl.): 218-229.

Green, R. E., Rocamora, G. & Schäffer, N. 1997. Populations, ecology and threats to the Corncrake *Crex crex* in Europe. *Vogelwelt* **118**: 117 – 134.

Gärdenfors, U. (ed.) 2005. *Rödlistade arter i Sverige 2005 – The 2005 Red List of Swedish Species*. ArtDatabanken. SLU. Uppsala.

Hampe, A. & Petit, R. J. 2005. Conserving biodiversity under climate change: the rear edge matters. *Ecology Letters* **8**: 461–467.

Höglund. J. 2009. *Evolutionary Conservation Genetics*. Oxford University Press. Oxford.

Jägerskiöld, L. A. & Kolthoff, G. 1926. *Nordens fåglar*. Stockholm.

Koffijberg, K. & Schäffer, N. (Compilers) 2006. *International Single Species Action Plan for the Conservation of the Corncrake* Crex crex. CMS Technical Series No. 14 & AEWA Technical Series No. 9. Bonn. Germany.

Lande, R. 1988. Genetics and Demography in Biological Conservation. *Science* **241**: 1455-1460.

Larsson, J. K., Jansman, H. A. H., Segelbacher, G., Höglund, J. & Koelewijn, H. P. 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.

Luikart, G. & Cournet, J. M. 1998. Empirical Evaluation of a Test for Identifying Recently Bottlenecked Populations from Allele Frequency Data. *Conservation Biology* **12**: 228-237

Miller, C. R. & Waits, L. P. 2003. The history of effective population size and genetic diversity in the Yellowstone grizzly (Ursus arctos): Implications for conservation. *PNAS* **100**: 4334–4339

Myers, N. 1987. The extinction spasm impending: synergisms at work. *Conservation Biology* **1**: 14-21

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**: 583-590.

Ottvall, R. 1999. The Corncrake (Crex crex) in Sweden. In: Schäffer, N. & Mammen, U. (eds.) (1999): *Proceedings International Corncrake Workshop 1998*. Hilpoltstein, Germany.

Park, S. D. E. 2001. Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection. [Ph.D. thesis] University of Dublin.

Peakall, R. & Smouse, P.E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*. **6**: 288-295.

Pettersson, T. 2007. *Åtgärdsprogram för kornknarr, 2007–2011*. Naturvårdsverket. Stockholm.

Piry, S., Luikart, G. & Cournet, J. M. 1999. BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity* **90**: 502-503.

Primmer, C. R., Saino, N., Møller, A. P. & Ellegren, H. 1998. Unraveling the Processes of Microsatellite Evolution Through Analysis of Germ Line Mutations in Barn Swallows *Hirundo rustica*. *MolecularBiology and Evolution* **15**:1047–1054.

Risberg, L. 1987. Faktablad: *Crex crex* – kornknarr. Modified: Lennart Risberg 1994, Richard Ottvall 2002, Åke Berg & Thomas Pettersson 2006. ArtDatabanken. SLU 2006.

Risberg, L. 1988. Kornknarr Crex crex L. In: Andersson, S. (ed.) *Fåglar i jordbrukslandskapet*. Vår Fågelvärld, suppl. **12**.

Schäffer, N. & Green, R. E. 2001. The Global Status of the Corncrake. In: Drury, I. (ed.) *RSPB Conservation Review* **13**.

Sefc, K. M., Payne, R. B. & Sorenson, M. D. 2003. Microsatellite amplification from museum feather samples: effects of fragment size and template concentration on genotyping errors. *The Auk* **120**: 982–989.

Spielman, D., Brook, B. W. & Frankham, R. 2004. Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences of the United States of America* **101**(42): 15 261-15 264.

Svensson, S., Svensson, M. & Tjernberg, M. 1999. *Svensk fågelatlas*. Vår fågelvärd, suppl. 31. Stockholm.

Tyler, G. 1996. The Ecology of the Corncrake with special reference to the effect of mowing on breeding. [PhD thesis] University of Ireland/University College Cork. In: Schäffer, N. & Mammen, U. (eds.) (2001): *Proceedings International Corncrake Workshop 1998*. Hilpoltstein, Germany.

Wettstein, W. 2002. Population structure. dispersal and migration of Corncrakes (*Crex crex*) in Europe. Progress report November 2002.

Wettstein, W. & Schäffer, N. Population structure, dispersal and migration of Corncrakes (*Crex crex*): genetic and chemical information obtained from feathers. Research project proposal. BirdLife International Corncrake Conservation Team.

Appendix 1

Samples from the Swedish Museum of Natural History. * indicates samples from gift 25 2008, while the remaining samples are from gift 32 2008.

Registration nr	Collection site	Collection date	Sample type
50537	Uppland	1863 05 03	Toe pad
50568	Uppland	1867 05 20	Toe pad
50830	Uppland	1891 05 14	Toe pad
51067	Skåne	1895 07 11	Toe pad
569986*	Värmland	1898 06 05	Toe pad
569983*	Skåne	1900 09 13	Toe pad
51220	Skåne	1900 09 13	Toe pad
51879	Gotland	1907 10 05	Toe pad
52942	Jämtland	1919 06 19	Toe pad
53396	Småland	1922 06 04	Toe pad
55760	Uppland	1931 05 16	Toe pad
55886	Uppland	1931 09 19	Toe pad
569984*	Uppland	1932 08 27	Toe pad
57568	Öland	1938 09 16	Toe pad
569985*	Södermanland	1940 06 01	Toe pad
59253	Uppland	1946 05 17	Toe pad
73300	Uppland	1954 06 18	Toe pad
680366*	Öland	1968 09 30	Toe pad
986432*	Öland	1996 08 12	Tissue
986363*	Öland	1997 06 17	Tissue

Appendix 2

The nine microsatellite loci used in this study and their respective primer sequences (from Gautschi *et al*. 2002). Size ranges of PCR products from this study are measured in base pairs. Ranges found by Gautschi *et al*. (2002) are also included within parenthesis signs. Italic letters before each forward primer represents the fluorescent labelled dye used in the amplification reactions. Crex1 was, due to low efficiency, excluded from the final analysis, wherefore no obtained size ranges from this study are shown.

Locus	Primer sequence (5' to 3')	Repeat motif	Size range
Crex 1	*(6-FAM)*CACTGTTCCTTTGGAACTTTCTC	$(GT)_9$ AT $(GT)_8$	(135-176)
	TAACCCCAGGGATCATTTTG		
Crex2	*(HEX)*GTGTCTCAGGCAGCACAGAA	$(GT)_5$ AT $(GT)_6$ CT $(GT)_7$	85-119
	AGCAGGGCAGGACCCATT		(81-117)
Crex4	*(6-FAM)*CACAGGCTGGCACAGTTG	$(CA)_{10}$ CTCATG $(GA)_2$ GC $(GT)_2$	123-159
	GTGCGGTTGTTCGATGTG	G $(CA)_2$ $(C)_4$ CATGGG $(CA)_3$	(124-167)
		GGCTGG $(CA)_2$ $(CG)_2$ $(CA)_{13}$	
Crex6	*(HEX)*CGCCCAAGTTGTCTTCATC	$(AC)_{22}$	75-139
	ACAGTGCTGCAGGGGAAG		(70-128)
Crex7	*(6-FAM)*TCTCTCCAAGGGAACAGCTC	$(GA)_{23}$ GG $(GA)_{10}$	110-142
	TATTTGGCCTGAGCTGCAA		(109-154)
Crex8	*(HEX)*GAACCAGAGCAAAGGAGGAG	$(AC)_{12}$	175-209
	TCCACATCTTCCCATCACTG		(180-204)
Crex9	*(NED)*GCCAGGGAAAGATGGTTTTC	$(CA)_{11}$ TA $(CA)_4$	79-121
	AATGATGCTCCTGGAGATGG		(76-116)
Crex11	*(6-FAM)*CACCTGGTCAAGTAAGCAACC	$(CA)_{27}$	81-141
	GCTTGCATAACCTGTGCTTG		(77-134)
Crex12	*(HEX)*CTAATGGGGTTTTTGGTTGG	$(CA)_9$	120-134
	GACCCGATGATCTCTTGAGG		(113-131)