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Abstract
The objective of the study was to establish and refine a method for the genomic characterization of European hedgehogs in Denmark using the second-generation genotyping technique, genotyping by sequencing (GBS). Single nucleotide polymorphisms (SNPs) were filtered with a read coverage between 20 - 100 and a maximum number of missing data of 25 %. Individuals with > 25 % missing data were removed yielding a total of 2.4 million SNPs, and after filtering for Minor allele frequency (MAF) >1 %, 2902 SNPs remained. Approximately half of the individuals analysed contained less than 75% of the selected SNPs, and were removed, resulting in a sample size of 30. We estimated inbreeding coefficients (F), observed (H_O), expected (H_E) and unbiased expected (uH_E) heterozygosity and the percent of polymorphic loci (P%). We tested for deviations from Hardy-Weinberg equilibrium (HWE) and patterns of isolation by distance (IBD). We assessed the genetic structure of the sampled individuals based on a Bayesian clustering method, and tested for recent population expansion or decline. We found a P% = 94.5%, a uH_E and H_E of mean ± SE; 0.31 ± 0.04 and 0.30 ± 0.02, respectively and an H_O of 0.290 ± 0.03. The heterozygosity deficiency was reflected in a positive F-value; 0.1 ± 0.01 and a significant deviation for HWE ($p < 0.05$). The Mantel test for association between the genetical and geographical distances of populations was not significant ($b = 0.007$, $R = 0.145$, $p > 0.05$). The significant and positive F-value found, was explained by inbreeding, genetic substructure and low effective population size (N_e) which are all consequences of habitat fragmentation. We failed to detect recent signs of a population bottleneck or expansion. Further studies on a larger scale are needed to obtain a general view of the conservation status of the Danish hedgehog population.

Keywords: Structure; SNPs; Bottleneck;Effective population size; European hedgehog;*Erinaceus europaeus*;Habitat fragmentation;

Introduction

The western European hedgehog (*Erinaceus europaeus*), is a hedgehog species found on the British Isles and Continental Europe, from Iberia and Italy northwards into Scandinavia, as well as on New Zealand. It is a generally common and widely distributed species that can survive across a wide range of habitat types (Morris, 2014; Reeve, 1994). However, previous research on both national and local scale has documented decline or indicated concerns for decline of the hedgehog populations in several western European countries (Hof and Bright, 2016; Krange, 2015; Müller, 2018; SoBH, 2011; 2015; 2018; van de Poel et al., 2015; Williamset al., 2018). There is currently no data to indicate the status of the Danish population of hedgehogs, but nonetheless, the situation may be similar in Denmark, due to comparable habitat fragmentation, landscape structure, farm management practices and climate in the western European countries. The suspected reasons for the decline are habitat loss and habitat

fragmentation, intensified agricultural practices, road traffic accidents, molluscicide and rodenticide poisoning and badger predation (Brakes and Smith, 2005; Dowdinget al., 2010a; Dowding et al., 2010b; Haighet al., 2012; Hof and Bright, 2010; Hubertet al., 2011; Huijser and Bergers, 2000; Pettettet al., 2017; SoBH, 2011; Younget al., 2006).

Hedgehogs are nocturnal and non-territorial and can travel up to 2-3 km per night. Home ranges of adult hedgehogs are generally estimated to be 20-30 ha for males and 10 ha for females, expanding temporarily during the mating season (Morris, 2014). Juvenile hedgehogs do not disperse far from their natal area when reaching independence (Sæther, 1997), and adult hedgehogs seem to remain in the same area throughout their lives (Reeve, 1994). Previous studies furthermore indicate that relocated individuals do not disperse very far even when released into an unfavourable habitat (Doncasteret al., 2001).

During the past 20-30 years, the rehabilitation of sick, orphaned and injured wild hedgehogs has become an established practice in many western European countries. Denmark has several working hedgehog rehabilitation centers, where volunteers care for the hedgehogs and release them back into the wild, when they have recovered. The extent of hedgehog rehabilitation in Denmark is quite comprehensive, and there has been a tendency to transport hedgehogs over barriers such as seas for rehabilitation, and to release individuals in foreign habitats. Only recently have the Danish authorities established legal frameworks and monitoring programs for the practice of wildlife rehabilitation.

Genetic investigations of European hedgehogs have previously been conducted using microsatellites (Becher and Griffiths, 1997; 1998; Berggren et al., 2005; Bolfikova and Hulva, 2012; Bolfikova et al., 2013; Bolfikovaet al., 2017; Braakeret al., 2017; Fraseret al., 2012; Henderson et al., 2000; Moran et al., 2009; Santucci et al., 1998; Seddon et al., 2001). In this study the use of a genotyping by sequencing (GBS) (Elshireet al., 2011;Narum et al., 2013; Nørgaard et al., 2017; Pellegrino et al., 2016; Pertoldi and Randi, 2018) was adapted and optimized for the genetic analysis of European hedgehogs.

The flourishing discipline of citizen science, defined as the involvement of citizens from the non-scientific community in academic research, has gained increasing importance in the field of conservation biology. Citizen science provides researchers with an opportunity to obtain information that would otherwise be impossible to collect due to time and resource constraints, and offers motivated citizens an opportunity to contribute to scientific understanding and conservation through voluntary biological monitoring (Chandler et al., 2017; Conrad and Hilchey, 2011; Tulloch et al., 2013).The collection of genetic samples for the present study was successfully based on a citizen science approach.

The main aims of the experiment were: (1) To provide a set of SNPs, which can be used for investigating the genetic structure and variability of the European hedgehog on a broader scale (2) to evaluate the patterns of the genetic diversity distribution in the population of Danish hedgehogs in Jutland south of the Limfjord (Figure 1) and (3) ascertain the historical changes in their effective population size (N_e) through genetic signatures.

Materials and methods

The genetic samples were obtained as part of a nationwide citizen science project in Denmark, where volunteers were encouraged to collect dead hedgehogs, from May to December 2016, record the date and location of the find, and deliver the hedgehog carcasses to one of 26 collection stations distributed nationally. A total of 697 dead hedgehogs were collected. The hedgehog carcasses were stored at -20 °C. They were subsequently necropsied, and tissue samples from skin and muscle were extracted for the present research. Afterwards, the samples were stored at -20°C. The subsample used in this study was based on tissues from 62 hedgehogs collected on the Jutland Peninsula in southern Denmark.

Sample Preparation

DNA was extracted from 1-2 g of hedgehog tissue using the DNeasy Blood and Tissue kit (QIAGEN, Germany) following the manufacturer's instructions. The DNA samples (yield >0.5 mg/g tissue with

minimal sign of degradation) were digested with *Sau96I* (NEB) and ligated to adapters following the GBS protocol developed previously (Elshireet al. 2011). The ligated samples were pooled into 4 different pools and purified with AMPure XP beads (Beckman Coulter, USA). PCR amplification was performed on the 4 sample pools in 50 µL volumes containing approximately 50 ng pooled DNA using the Phusion High-Fidelity PCR kit (Thermo Scientific, USA) with the following program: 72 °C in 5 min, 98 °C in 30 sec, followed by 20 cycles of 98 °C in 10 sec, 66 °C in 30 sec and 72 °C in 30 sec, with a final extension at 72 °C in 5 min. The pools were then purified with AMPure XP beads (Beckman Coulter, USA) after which the DNA concentration was determined by Qubit (Thermo Scientific, USA) and visualized on the TapeStation 2200 using a D1000 Screen Tape (Agilent, USA). Paired-end 2x151 bp sequencing was run on the Illumina HiSeq X platform at Admera Health (South Plainfield, NJ, USA).

Filtering Raw Sequence Data, Mapping and SNP Calling

The i7 barcodes of the dual-barcoded sequenced reads were first de multiplexed with bcl2fastq2 version 1.0.0 (Illumina, Inc., San Diego, CA, USA) using zero mismatch allowance. Fastq-multx version 1.02.772 (https://github.com/brwnj/fastq-multx) was then used with one mismatch allowance to de multiplex the custom i5 barcodes (Elshireet al., 2011) and to remove the barcode sequences from the sequenced reads. Adaptor contamination and quality trimming was performed using Trim Galore with default parameters (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore). Burrows-Wheeler Aligner (http://bio-bwa.sourceforge.net/) (BWA) was used to align the reads against the European hedgehog (*Erinaceus europaeus*), EriEur 2.26 reference genome (http://hgdownload.cse.ucsc.edu/downloads.html). Reads with a mapping quality of at least 30 were kept. Variants were called using The Genome Analysis Tool Kit's Haplotype Caller (https://software.broadinstitute.org/gatk/documentation/tooldocs/3.80/org_broadinstitute_gatk_tools_walkers_haplotypecaller_HaplotypeCaller.php). Joint genotyping was performed using Genotype GVCFs(https://software.broadinstitute.org/gatk/documentation/tooldocs/3.80/org_broadinstitute_gatk_tools_ walkers_variantutils_GenotypeGVCFs.php). Initial filtering was performed using Select Variants(https://software.broadinstitute.org/gatk/documentation/tooldocs/3.80/org_broadinstitute_gatk_tools_walkers_variantutils_SelectVariants.php) and filtered for SNPs, bi-allelic sites, and mapping quality > 30 (DePristoet al., 2011). Minor allele frequency (MAF) was estimated from the read coverage, and SNPs were filtered on a minimum MAF of 1 % (average variant allele frequency <0.99 and >0.01). Additionally, SNPs were filtered with a read coverage between 20 and 100 and a maximum number of missing data of 25 %. Individuals with more than 25 % missing data were removed.

Population Genetic Variability and Structure

Inbreeding coefficient (F) observed (H_O), expected (H_E), unbiased expected (uH_E) heterozygosity and the percent of polymorphic loci (P%), were estimated using GENALEX v. 6.5 (Peakall and Smouse, 2012). The software GENEPOP 3.4 was used for testing for deviations from Hardy-Weinberg equilibrium (HWE) (Raymond and Rousset, 1995). GENALEX v.6.5 (Peakall and Smouse, 2012) was also used for checking if a pattern of isolation by distance could be found within every population investigated (Mantel, 1967). The geographic distance connecting samples was represented by Euclidean (linear geographic) distances computed in QGis(QGIS Development Team, 2019). Population genetic structure of the sampled individuals (N = 30), was assessed based on a Bayesian clustering method, implemented in the STRUCTURE v. 2.3.; 10 independent runs of K = 1- 10 were carried out on the populations with 10^6 Markov chain Monte Carlo (MCMC) iterations and 10^5 burn-in period on the basis of independent allele frequencies and admixture ancestry model (Pritchard et al., 2000).

Assessment of the Demographic History

Signature of a recent abrupt decline in the population size was evaluated by the program BOTTLENECK v. 1.2, after 10.000 iterations under infinite allele model (IAM) according to an excess of the heterozygosity and deficiency of the rare alleles (Luikart and Cornuet, 1998; Luikart et al., 2010).

Results

Genotypes

A total of 2.4 million SNPs were found. Following filtering for MAF >1 % estimated from read coverage, maximum missing data of 25 %, and read coverage between 20 and 100, 2902 SNPs remained. Approximately half, 32 of the original 62, individuals that contained less than 75 % of the selected SNPs were removed, resulting in 30 individuals remaining for the further investigation. See Figure 1 for an illustration of the locations of the subsample 30 individuals used in the study.

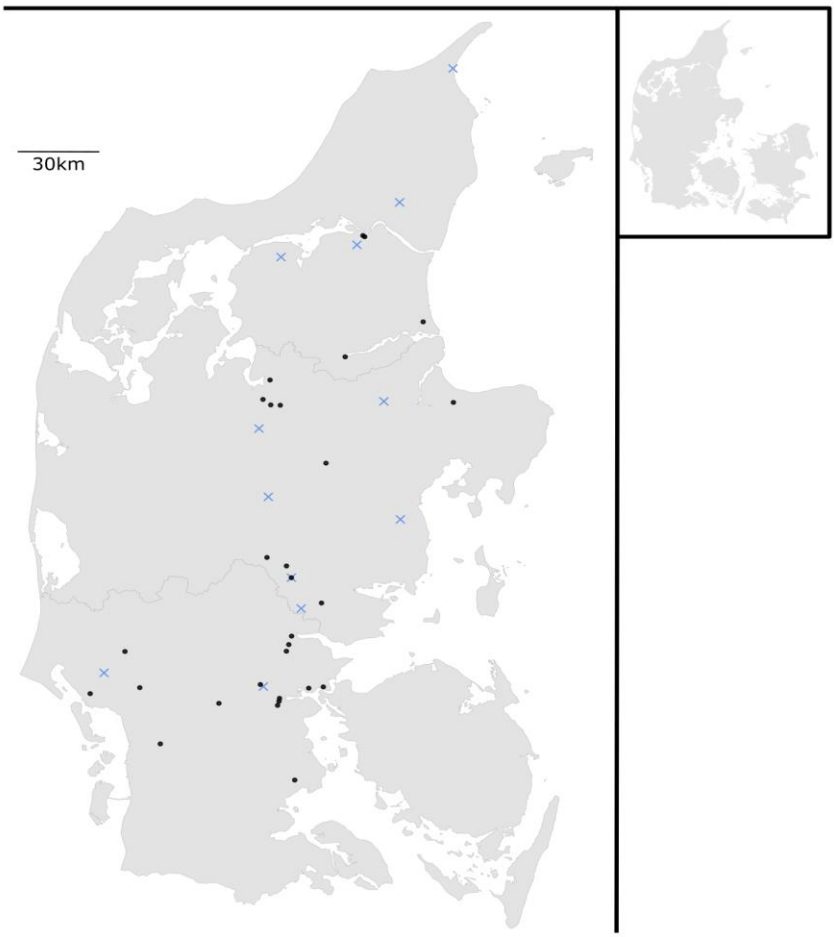

Figure 1. Map of Denmark indicating the sampling locations (black dots) of the subsample of 30 hedgehogs collected on the peninsula of Jutland, Denmark and *the locations of the twelve hedgehog collection stations (blue crosses) situated in Jutland, Denmark.*

Genetic Variability and Structure

The level of polymorphism found was high; P% = 94.5 %, the$u H_E$ and H_E estimated were quite similar; mean ± SE = 0.31 ± 0.04 and 0.30 ± 0.02, respectively, whereas the H_O was lower; 0.290 ± 0.03. The heterozygosity deficiency is reflected in a positive F-value; 0.1 ± 0.01 and a significant deviation for HWE ($p < 0.05$).

The Bayesian clustering of the genotyped data assigned the highest posterior probability; Estimated Ln Prob of Data = -147679.1, Mean value of ln likelihood = -147032.9, Variance of ln likelihood = 810.4, for K = 1.

The Mantel test for association between the genetic and geographical distances of populations was not significant (b = 0.007, R = 0.145), indicating that only about 2.1% (*i.e.* $R^2 = 0.145^2 = 0.021$) of the genetic divergence was explained by geographical distance.

Population Bottlenecks and Expansion

The population investigated failed to show a significant deficiency or excess in heterozygosity, which could indicate expansions or reduction in population size, respectively ($p > 0.05$).

Discussion

The level of genetic variability in the present study ($H_E = 0.30$ and $H_O = 0.29$) was considerably low compared to the mean $H_E = 0.62$ and $H_O = 0.44$ based on 41 European hedgehogs in a study conducted by Curto et al. (2019) using SNPs markers. The level of genetic variability was also lower than the variability found using microsatellites markers in the same study: $H_E=0.51$ and $H_O = 0.35$, likely because their study used individuals representing different countries across the European continent and thereby, was conducted on a much broader scale. The island structure of Denmark furthermore limits the gene flow. However, the individual hedgehogs chosen for the present study all derive from the peninsula of Jutland, which is connected to Germany, which could lead to an increased gene flow compared to the rest of Denmark, which consists of islands.

The low level of genetic variability and the significant and positive F-value, which indicate a significant deviation from HWE, are probably explained by inbreeding, genetic substructure and low N_e which can be due to habitat fragmentation, the limited dispersal behaviour of hedgehogs and relocation of rehabilitated hedgehogs (Becher and Griffiths, 1998; Bolfikova and Hulva, 2012; Braakeret al., 2017).

Despite the deviation from HWE in the present study, the STRUCTURE analysis failed to find further substructuring and the Mantel test failed to find an isolation by distance effect. The low R^2 value is not due to low power of the test as the sample size utilised for the regression analysis is large. The reasons could be due to the low level of genetic variability found in this investigation which have probably cancelled out the Wahlund effect which produce a heterozygosity deficiency due to the lack of panmixia. The relatively low number of individual hedgehogs included in the analysis (30 individuals after filtering) could also account for the lack of statistical power (and thereby lack of significance) in our analyses, especially given the low level of genetic variability among the sampled Danish population.

The software BOTTLENECK 1.2. failed to detect signs of population bottlenecks or expansions. However, the software is only able to detect recent decreases or increases in population size, within 0.2 N_e to 0.4 N_e generations. We therefore cannot reject the hypothesis that the Danish hedgehog population has declined before the scope of 0.2 to 0.4 N_e generations.

Conclusion

We developed a successful and effective method for the genomic characterization of European hedgehogs in Denmark using the GBS technique with 2.4 million detected SNPs. The low level of genetic variability and the significant and positive F-value found in the subsample of Danish hedgehogs may be explained by inbreeding, genetic substructure and extremely low N_e, which could be due to habitat fragmentation, relocation of rehabilitated hedgehogs, the limited dispersal behaviour of hedgehogs and the general island structure of Denmark. We failed to detect recent signs of a population bottleneck or expansion (within 0.2 N_e to 0.4 N_e generations). However, this does not rule out the possibility that the hedgehog population of Jutland has previously undergone a decline.

By integrating population genetics and conservation biology, further studies with a larger sample size, representative of the general hedgehog population of Denmark, would enable a conclusion on the conservation status of the Danish hedgehog population. A relevant future focus of research would be to compare individual heterozygosity with measures of health e.g. parasite load, dental health, toxicology and presence/absence of cancer, to understand how the low genetic variability detected in the present study affects hedgehogs at an individual level.

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Authors' Contributions

Sophie Lund Rasmussen: Original idea, managing and gathering the collection of samples, extraction of samples, data analyses, writing of the manuscript as first author and corresponding author.

Erika Yashiro and Jeppe Lund Nielsen: Designing the molecular analysis and editing of the manuscript.

Elsa Sverrisdóttir, Kåre Lehmann Nielsen, Mie Bech Lukassen and Torben Asp: Bioinformatics.

Cino Pertoldi: Supervising laboratory work, bioinformatics, data analyses, writing of manuscript, responsibility for supervision of the writing of the first author in the role as last author.

Ethics

No ethical issues to declare.

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