



Sveriges lantbruksuniversitet
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Environmental Studies

Golden Eagle (*Aquila chrysaetos*) genomics across Scandinavia – Population structure and effects of marker selection

Genomik hos Kungsörn (Aquila chrysaetos)

– Populationsstruktur och effekter av markörselektion

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Master's thesis • 30 credits

Jägmästarprogrammet

Examensarbete/Master's thesis, 2018:3

Department of Wildlife, Fish, and Environmental Studies

Umeå 2018

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Credits: 30 credits

Level: Second cycle, A2E

Course title: Master degree thesis in Biology at the Department of Wildlife, Fish, and Environmental Studies

Course code: EX0633

Programme/education: Jägmästarprogrammet

Place of publication: Umeå

Year of publication: 2018

Title of series: Examensarbete/Master's thesis

Part number: 2018:3

Online publication: <https://stud.epsilon.slu.se>

Keywords: *Aquila chrysaetos*, rad-seq, marker selection, hardy weinberg, population structure

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Abstract

Rapid development in DNA-sequencing has led to increased use of single nucleotide polymorphisms (SNPs) as molecular markers in wildlife genetic studies. RAD-sequenced SNP-markers can produce large datasets that are successfully used in studies on animal population structure. The high number of markers does however create both possibility and need for deliberate marker selection. Removal of markers outside Hardy Weinberg Equilibrium (HWE) to avoid sequencing error, might exclude informative markers, and marker selection at different minor allele frequencies (MAF) can generate differences in population structure. In the present study SNP-markers are used to study basic biology in Scandinavian Golden eagles, and by searching for genetic structure among populations, addresses questions of natal philopatry and interspecific gene flow. Simultaneously, effects of marker selection on population structure are examined. 8710 SNPs from 96 Golden eagles were analysed for population genetic structure using principal component analysis (PCA). Marker selections were based on HWE and different windows of MAF. Results show genetic structure indicating limitations in gene flow between mainland Sweden and the island of Gotland. Gradual east-west structure was found in mid Sweden and Norway, possibly related to habitat type. Markers outside HWE were important in detecting population structure, and inclusion of these in the PCA outweighed any effect of possible sequencing error. Different MAF-windows mainly affected the degree of separation between genetic clusters, but weak indices of some structuring only visible at certain MAF-windows were also found. These results support previous findings on natal philopatry of golden eagles and question the use of only HWE to identify sequencing error.

Keywords: aquila chrysaetos, rad-seq, marker selection, hardy weinberg, population structure

1 Introduction

Population genetic principles and molecular genetic data have become established tools in conservation and management of wildlife species (Frankham, Briscoe and Ballou, 2002). Genetic data are often used when studying population structure and to define units of conservation (Moritz, 1999). This type of studies can also give insights on animal dispersal patterns (Rudnick et al., 2008; Norman and Spong, 2015; Ogden et al., 2015) and connectivity between populations (Craig et al., 2016).

1.1 RAD-sequencing & SNP's

The last decades have shown rapid development in DNA sequencing, and in particular the use of restriction site-associated DNA sequencing (RAD-Seq; Davey and Blaxter 2010). A method which uses restriction enzymes to target a subset of the genome, making it possible to sequence large datasets time- and cost efficiently, and unlike many other methods requires no prior genomic knowledge of the species studied (Andrews et al., 2016).

This high throughput technology has led to an increase in the use of single nucleotide polymorphisms (SNP's) as the genetic marker of choice in many studies (Grover and Sharma, 2016). SNP's are variations at a single position in a specific DNA-sequence among individuals, with only the four bases of DNA as possible alleles, making the resolution per marker low compared to microsatellites (Liu et al., 2005; Hauser et al., 2011). The lower resolution is however balanced by the ease to obtain a high number of markers, resulting in the possibility and need for strict filtering of the dataset, thus preserving only data of the highest quality (Norman, Street and Spong, 2013). Recent studies using SNPs to analyse population structure shows good results across several species (Doyle et al., 2016; Pellegrino et al., 2016; Norman et al., 2017; von Thaden et al., 2017).

The combination of several highly informative SNP markers into panels, allows for genotyping of samples with low quality DNA, such as non-invasively collected hair and faecal matter (Morin, Luikart and Wayne, 2004; Morin and McCarthy, 2007; Norman and Spong, 2015). By pedigree reconstruction from such data, population estimates can be done, successful examples are estimates for populations of brown bear, *Ursus arctos* (Spitzer et al. 2016) and lions, *Pantera leo* (Creel and Rosenblatt 2013). Similar pedigrees have also been shown as a useful tool in studies on dispersal in brown bear (Norman and Spong 2015) and mating behaviour in lions (Jansson 2017). SNP markers can thus be argued as an important tool in population ecological research.

1.2 Population structure and marker selection

With high- throughput technologies like RAD-Seq, finding erroneous data such as sequencing errors can prove difficult. One method of filtering out possible sequencing errors is to select only markers that fall within the Hardy Weinberg Equilibrium (HWE, King et al. 2007), as markers with sequencing error tend to deviate from HWE (Hosking et al., 2004). However, this method poses a problem when studying population structure, as part of the HWE definition is a population subject to no selection, and where mating is random. By removing markers outside of HWE, we might lose important information on population structure, as these might be markers under selection that create the population substructures. So as the identification and removal of genotyping errors is important, it should not be done at the expense of detecting population structure.

One method of detecting population structure in large datasets of SNPs is with the use of a principal component analysis (PCA, Jolliffe, 2014; Pellegrino et al., 2016). This method summarizes many variables into single components with a maximum amount of variance across the samples, and thus efficiently visualise variation among samples. With large datasets, the contribution of a single marker to the result of the PCA is therefore quite small, and the effect of single sequencing errors could possibly be negligible. Recent studies have shown there might be methods to differentiate between departures from HWE due to natural variability or genotyping error through selection by heterozygosity excess or deficiency (Chen, Cole and Grond-Ginsbach, 2017). But when using PCA and the number of markers is high enough, it might be possible to simply disregard HWE filtering.

Another issue to consider in the choice of markers is minor allele frequency (MAF). De la Cruz and Raska (2014) show how different population structures becomes

evident in analysis at different windows of allowed MAF. They argue for a scale effect, where rare variants with low MAF, are more likely to be shared only locally, and thus important in detecting local genetic structures. For SNP's with high MAF, all possible genotypes are more common, and thus more likely to be shared over long distances. While good for detecting large scale population structures, using only common markers might miss local variations. An additional argument for using different windows of MAF for these analyses, is that the combination of common and rare markers in a PCA, might dilute structures otherwise apparent using just one or the other (De la Cruz and Raska, 2014).

1.3 Golden eagle

The golden eagle (*Aquila chrysaetos*) is an apex predator with a distribution throughout much of the northern hemisphere, and among the most widespread of avian species (Watson, 2010). It is listed in the Annex 1 (species needing habitat conservational measures) of the EU Birds-, and Habitat directive (European Commission, 1992, 2009), and top predators like the golden eagle can be argued as important focal species in conservation work due to umbrella effects on other species (Sergio et al., 2006). It can be found mostly throughout Fennoscandia (Mullarney, Svensson and Zetterström, 2010) and in Sweden it is red-listed as near threatened (Tjernberg, 2015). It is one of five large predators actively managed in Sweden, with issues of predation on both livestock and semi-domesticated reindeer (Naturvårdsverket, 2011, 2013) and also an important species of concern in the development of wind farms (Drewitt and Langston, 2006; Tjernberg, 2015).

Like many raptors, golden eagles can be difficult to study as they often reside in remote and rugged areas (Watson, 2010; Tjernberg, 2015), individuals can be hard to capture and even harder to resight. This is however an obstacle which partly can be overcome by genetics. From a single sampling event, genetic markers can be generated which can be applied in a number of ecological contexts as described above. These markers can also work as individual tags which allow for re-sighting through non-invasive sampling (Rudnick et al., 2008).

Scandinavian golden eagles have some of the largest reported home ranges worldwide, Moss et al. (2014) found a mean home range size of 226km², compared to the 16.73km² reported by Crandall et al. in Montana, U.S.A. (2015), using similar methods for North American golden eagles. These large home ranges are somewhat reflected in juvenile dispersal distance, where high latitude golden eagle populations show long distance seasonal movements (McIntyre, Douglas and Collopy, 2008b), and first year dispersals of up to 1500 km has been found in Scandinavia (Nygård

et al., 2016). As these are seasonal movements, juvenile birds tend to return to their natal areas in spring (McIntyre, Douglas and Collopy, 2008b; Nygård et al., 2016), but it is unclear if this behaviour continues into pre-adulthood and as they reach breeding age. Adult breeding birds do show high fidelity to their home range, and breeding couples often return to the same core areas year after year (Marzluff et al., 1997; Watson, 2010; Tjernberg, 2015), but whether the step from juveniles into a breeding population takes place in their natal area or not, the knowledge is limited. If adult birds return to their natal areas to breed, this should be visible in the population genetic structure, as gene flow would be limited to within the natal area of the birds.

Recent studies on natal dispersal distance of Golden eagles in the U.S.A. (Millsap et al. 2014) and population genetic structure in the U.S.A. (Doyle et al. 2016) and Scotland (Ogden et al. 2015) gives some support for the idea of natal philopatry in golden eagles. Conflicting results from Canada and the U.S.A., have been shown by Craig et al. (2016) pointing towards high connectivity and low genetic structures. These studies all argue for further exploration on the subject, as there are difficulties such as comparing migratory and resident populations, and need for additional geographic sampling and increased genomic analyses.

If genetic structures can be found in a relatively small area such as the outer islands of Scotland (Ogden, 2015), and in populations shown to seasonally roam over much of the North American continent (McIntyre, Douglas and Collopy, 2008a; Doyle et al., 2016), what is the genetic structure of Scandinavian eagle populations? Here populations are spread over vast distances with a large latitudinal gradient, and some show large seasonal movements. Do these movements result in high connectivity and increased gene flow between populations, or is there further support for natal philopatry?

1.4 Aim

In this study, I address golden eagle philopatry by searching for possible genetic structuring among populations in Fennoscandia. With the use of SNP-markers generated from golden eagles sampled in Denmark, Finland, Norway and Sweden, I aim to give a first insight into population genetic structure in these areas. This could give further understanding in the question of natal philopatry in golden eagles, and if there is evidence for gene flow limitations between populations, provide valuable information for management and further research. Through different

selection of markers, I aim to simultaneously examine possible effects of HWE-filtering and MAF on marker resolution and potential population structures.

2 Method

2.1 Study area and sample collection

This study is performed as part of the first steps towards the development of non-invasive monitoring tool for Scandinavian golden eagles in form of a SNP panel. Knowledge of differences in the genetic structure across these countries could thus provide useful information in this process. So as an addition to sampling golden eagle's in Sweden, samples were also requested from Denmark, Finland and Norway.

The majority of samples were collected through citizen science by members of civil organisations. 58 samples were taken as part of basic ecological monitoring programmes when banding juvenile birds at the nest. 28 adult eagles were captured by net and 7 additional samples were provided by the Swedish Governmental Veterinary Institution (Statens Veterinärmedicinska Anstalt, SVA), as part of disease monitoring programme for wildlife by examination of deceased animals. Three samples had insufficient data to determine sampling method, but were included in the genomic analyses anyway.

The study area spans from 55° N in Denmark to 70° N in Finnmark, Norway, a distance of roughly 1700km. The southern areas are dominated by farmland with patches of boreo-nemoral forest. The northernmost sampling area, in the county of Finnmark, Norway consists of a tundra landscape with proximity to the North Sea. Remaining northern areas are dominated by a boreal forest landscape, with mountain ranges along the Sweden-Norway border. Samples were also collected from the island of Gotland, separated from mainland Sweden by 80km of open sea. With a size of about 3000km², dominated by farmland, heaths and pine forest on

calcareous soil, it has one of the densest populations of golden eagles worldwide (Tjernberg, 2015).

Samples were split into seven regions by rough geographic features as seen in Figure 1; the mid Sweden and Norway samples were split into MidEast and MidWest, where MidWest is defined by a proximity to large mountain ranges, and MidEast dominated by lowland boreal forests. Swedish counties of Västerbotten and Norrbotten (VB+NB) had the highest number of samples and mainly in lowland boreal forest. Number of samples in each region can be seen in Table 1.

Table 1. *Number of samples in each sampling region.*

Region	No. Of Samples
Denmark & Skåne (DK+Skåne)	5
Finland	2
Finnmark	5
Gotland	7
MidEast	19
MidWest	17
Västerbotten & Norrbotten (VB+NB)	40
Unknown	1

For the regional population structure analysis only juvenile birds were included, as origin for adult birds could not be certain. All Finland samples were adults, and thus excluded from this analysis. One additional individual was also removed from the analysis because of unknown origin.

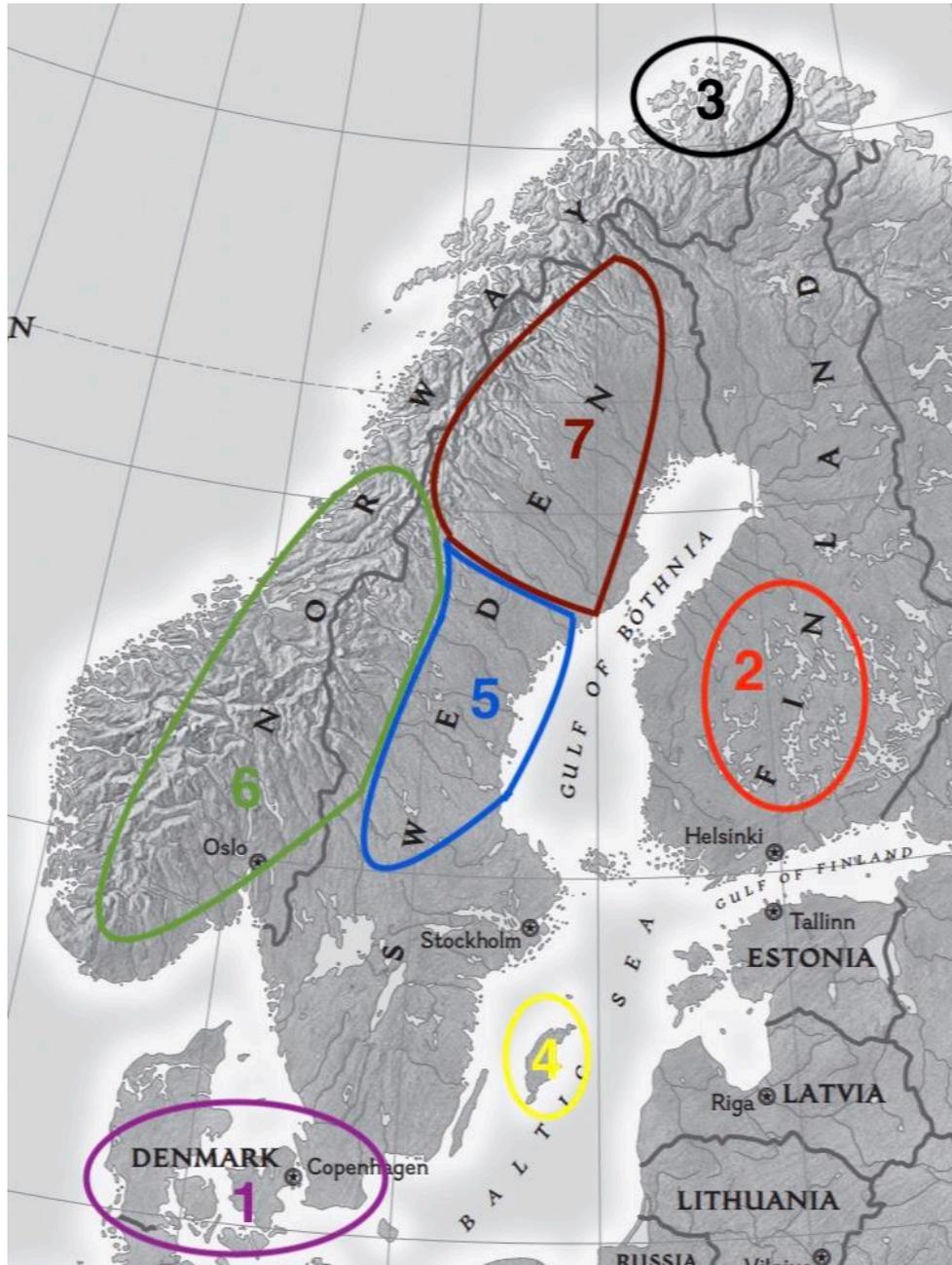


Figure 1. Sampling regions of Golden eagles 1; DK+Skåne, 2; Finland, 3; Finnmark, 4; Gotland, 5; MidEast, 6; MidWest and 7; VB+NB.

2.2 DNA extraction and genotyping by sequencing

Samples were digested using the restriction enzyme EcoR1, and fragments between 500-700 base-pairs were extracted before library construction. Genotype data from

96 individuals were then generated by paired end RAD-sequencing in a single lane on an Illumina HiSeq 2500 set to rapid mode. SNP screening was done in Stacks under default settings and only stacks holding a single bi-allelic SNP were kept.

2.3 Marker selection an analysis

For purpose of rationality and ease of data processing, only markers with one bi-allelic SNP were selected. To achieve good marker coverage of the sampled population, only markers present in 50% of the population (48 individuals or more) passed selection.

Deviations from HWE were calculated using Pearson's Chi-square goodness of fit test, with $df=1$ and $\alpha=0.05$. Thus, genotypic variations exceeding the chi-square critical value, has a probability of 95% or more to be due to deviation from HWE and not to chance. Markers with HWE deviation were split in two categories based on heterozygosity excess or deficiency compared to expected values.

To study effects of HWE and MAF on the population structure results 12 different marker selections were made. These were based on three windows of MAF, (<0.1 , $0-0.5$ and >0.4) and four different categories based on HWE (TRUE, TRUE+DEFs, TRUE+Exc & All). Where DEFs is markers with HWE deviation due to heterozygosity deficiency, and Exc is markers with HWE deviation due to excess heterozygosity as described by Chen, Cole and Grond-Ginsbach (2017).

Principal component analysis was performed for the different marker selections using the package SNPRelate in R.

3 Results

3.1 Markers

A total of 29361 SNP markers were found in Stacks and out of these 21959 contained a single bi-allelic SNP. SNP's found in 50% of the individuals or more counted to 8710. Number of SNP in the different selections based on MAF and HWE used for PCAs are shown in Table 2. A majority of the HWE deviations were due to heterozygosity deficiency, and for markers with low MAFs (0-0.1) all of the HWE deviations were due to deficiency. Total number SNPs with HWE deviation in all used markers were 1993 (22.9%).

Table 2. Number of SNP markers in selected categories

		1	5	6
	MAF	0-0.1	0.4-0.5	0-0.5
a	TRUE	1a (4247)	5a (383)	6a (6717)
b	TRUE+Defs	1b (5189)	5b (521)	6b (8509)
c	TRUE+Exc	1c (4247)	5c (476)	6c (6918)
d	HWE All	1d (5189)	5d (614)	6d (8710)

3.2 Population structure

Gotland samples create a distinct cluster separated from the rest, which is also true with the inclusion of adult birds. Mid Sweden and Norway samples indicate a gradual east west pattern, which becomes less clear with the inclusion of adult birds. Remaining samples display no clear population structures. Comparison of the Finnmark and Skåne-Denmark samples, separated by roughly 1700km, gives no indices of differences in genetic structure between these two areas (Figure 2 & 3).

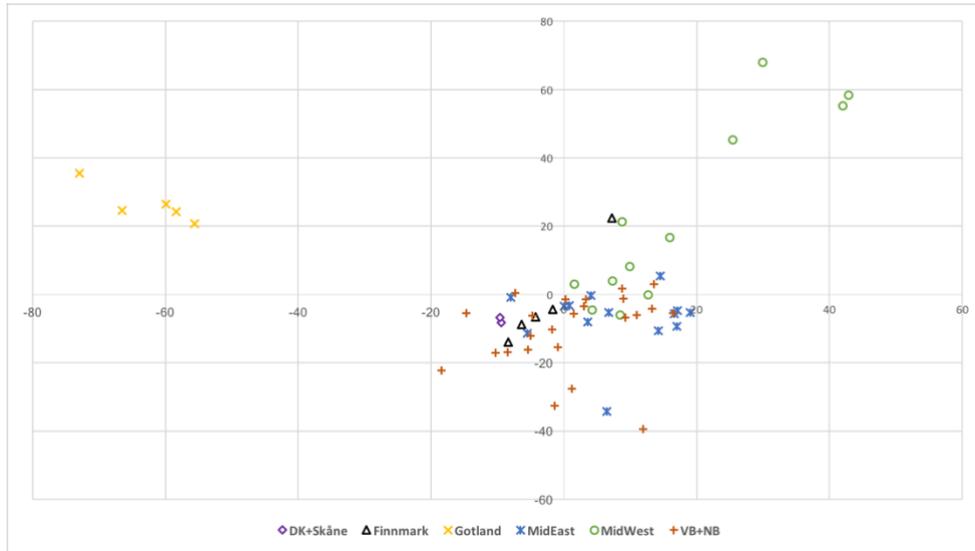


Figure 2. PCA based on all markers with no selection for MAF or HWE, showing Golden eagle population structure, including 62 juvenile birds from six regions.

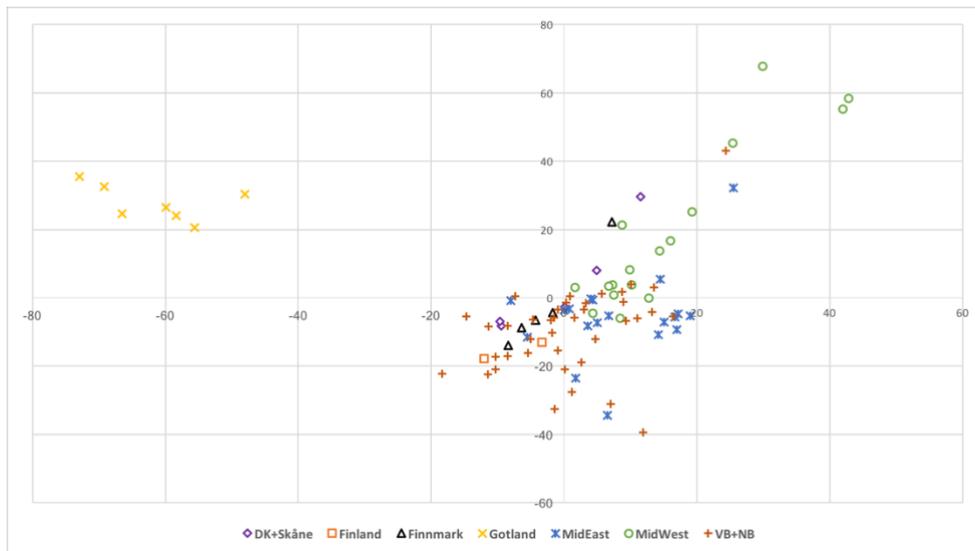


Figure 3. PCA based on all markers with no selection for MAF or HWE, showing Golden eagle pop. structure, including 95 adult and juvenile birds from the 7 regions.

3.3 Marker selection

Population structure did differ depending on HWE selection. PCA for markers within HWE showed different clustering, and some patterns of population structure was absent compared to PCA with inclusion of all markers (Figure 4, 6a & 6d).

Analyses of HWE TRUE and TRUE+Exc were identical (Figure 4, 6a & 6c), which was also the case for TRUE+Defs and All (Figure 4, 6b & 6d).

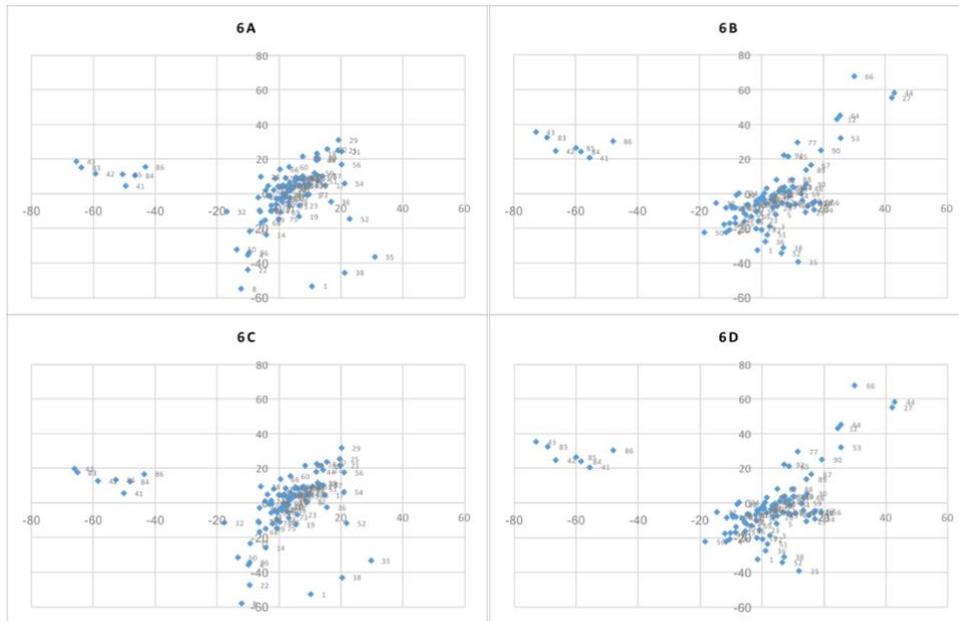


Figure 4. PCA showing pop. structure at different HWE selections at MAF 0-0.5. 6a: TRUE, 6b: TRUE+Defs, 6c: TRUE+Exc and 6d: All.

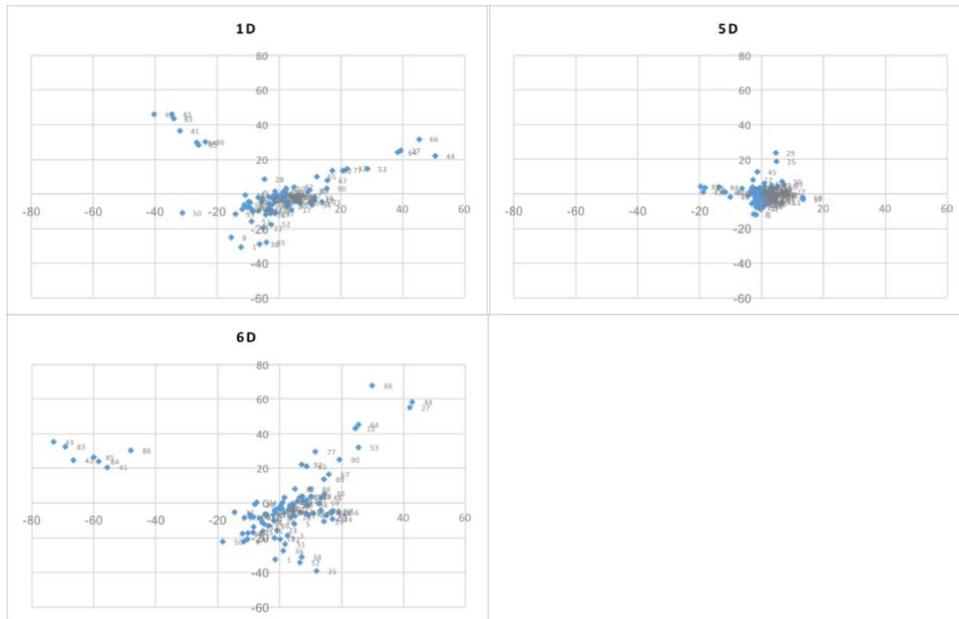


Figure 5. PCA showing pop. structure at different windows of MAF with no selection for HWE, 1D:0-0.1, 5D: 0.4-0.5 and 6D: 0-0.5.

PCA also showed differences in population structure depending on MAF. PCA for MAF windows of 0-0.1 and 0-0.5 (all), show similarly distinct clusters but with greater separation with the inclusion of all MAFs (Figure 5, 1d & 6d). Markers with high MAFs (0.4-0.5) provided less pronounced structures and with weaker separation between clusters compared to the others. Some individuals did stand out in the high MAF PCA that was not present in the other analyses (Figure 5).

4 Discussion

4.1 Population structure and natal philopatry

What appears like a distinctively different genetic structure in the Gotland eagles makes a strong argument for limitations in gene flow for this population. These are similar results as found by Ogden et al. (2015) on outer islands of Scotland. In addition to these results they also found reduced genetic diversity on the islands compared to mainland populations. This might also be the case for the Gotland population, but further sampling in this area would be needed before conclusions can be drawn on this subject. The genetic distinctiveness of the Gotland eagles does support the idea of natal philopatry here. But as an island population of a bird previously shown to avoid large waterbodies (Singh et al., 2016) it might be that dispersal is simply prevented by the 80km of open sea separating the island from mainland Sweden.

The gradual genetic difference between the MidEast and MidWest areas does to some extent conform to differences in habitat type. This indicates that there could be a possible limitation in gene flow between mountainous and lowland-forest dwelling populations, something which could be interpreted as evidence for natal philopatry. The lack of clear structures in North-South direction suggests higher connectivity between populations along this latitudinal gradient, which could be an indication that dispersal and gene flow is North-South biased. For more conclusive results on these structures and patterns of dispersal, additional sampling should be made. In addition, as some of the juvenile individuals included in the study were net-captured after fledgling, the true natal origin of these birds cannot be guaranteed.

4.2 HWE-filtering

The effects of marker selection on the results from the PCAs were clear, roughly similar patterns of population structure could be observed with or without the use of HWE-filtering (Figure 4). However, structures were more pronounced, and distinct genetic clusters included additional individuals when including markers with HWE-deviation. This supports the suspicion that simply testing for HWE to detect and remove sequencing errors, to some extent is at the expense of the results of the PCA. With a high number of markers, and for wide analyses such as a population structure PCA, the inclusion of markers with HWE-deviation rather seems to enhance results even with the inclusion of some possible sequencing error.

The division of HWE deviations into heterozygosity excess and deficiency as described by Chen, Cole and Grond-Ginsbach (2017) gave interesting results in the PCA. Analyses of markers within HWE (TRUE), and with the addition of deviations due to excess (TRUE+Exc) were identical (Figure 4, 6a & 6c). According to Chen, Cole and Grond-Ginsbach results (2017), deviations due to excess should contain a majority of the possible sequencing errors, and thus add erroneous data to the PCA. As this selection of HWE deviations did only add another 200 markers to the existing 6700 within HWE (Table 1), these results support the suspicion that the inclusion of a small amount of sequencing errors in a large dataset has no effect on the results of a PCA. The comparative analyses between markers within HWE with the addition of HWE deviations due to deficiency (TRUE+Def) and all markers, the results were also identical (Figure 4, 6b & 6d). This selection should exclude sequencing errors according to Chen, Cole and Grond-Ginsbach (2017) and here adds 2000 markers to the existing 6700 within HWE (Table 1). While this selection does provide additional information compared to using only markers within HWE, the results do not differ from the analysis using all markers. So as the inclusion of possible error seems to have no effect, neither does the exclusion of these errors seem to have effect compared to simply using all markers.

For large dataset PCAs, HWE filtering does seem to be at the cost of informative markers and provides no apparent gain to the results. This does not however make the need for precise removal of sequencing errors redundant. Smaller datasets, and studies targeted at more narrow subjects than population structure is probably more sensitive to the inclusion of sequencing errors. In those cases, refined methods of marker selections using HWE are probably useful.

4.3 MAF

The strongest effect of MAF-selection on the PCA-output was a scale effect. Markers with high MAF-values gave less pronounced structures, and the distinctively separated clusters found in the other analyses were absent or not as separated from the main cloud (Figure 5). One explanation for this could be that these selections had significantly lower number of markers compared to the others (Table 1). But this is not very plausible as there were still a high number of markers compared to similar studies (Doyle et al., 2016; Norman et al., 2017). These findings support De la Cruz and Raska (2014) who argues that common variants with high MAF are shared over longer distances and thus not very efficient in detecting local variations.

The PCA's at $MAF < 0.1$ and $MAF = 0-0.5$ showed quite similar structures but differed somewhat in the degree of separation between clusters compared to each other (Figure 5). This supports the idea that markers with low MAF might play an important role in detecting population structure, but are further enhanced with the inclusion of higher MAF-markers. De la Cruz and Raska (2014) argues for the importance of analysing at different windows of MAF when searching for population structures using PCA. The results from the different windows of MAF in this study, does give some weak indices of additional structuring, only visible at certain MAF and for a few individuals. But additional sampling and analysis would be needed for any further conclusions.

If using only markers with a high MAF when performing population structure PCA's, there might be a risk to exclude markers important in detecting these structures. One example of this could be where Norman et al. (2017) found no evident structures with PCA of brown bear in Scandinavia, but found pockets of clear genetic structure using the same data analysing relatedness. Their PCA was done using SNP markers with a mean MAF of >0.37 . Perhaps high MAF values could explain why these structures were not evident in their PCA?

4.4 Conclusion

These results show genetic structuring in the Scandinavian Golden eagle population, which could provide useful information for management decisions such as defining management units. The use of HWE filtering to exclude possible sequencing error in large dataset PCA's appears redundant, and even at the cost of important markers. The results on genetic structuring will provide important information in the next step of identifying markers suitable for SNP-panel development. This development

will hopefully lead to possibilities for non-invasive monitoring and sampling of the Scandinavian Golden eagle, which could provide efficient tools for management and research.

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Acknowledgements

Thanks to my supervisor Göran Spong for feedback and support, and also to Bastian Schifftaler, Helena Königsson and Navinder Singh for help with data, bioinformatics and analysis.

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