# Mate choice and patterns of adaptive variation in the Eurasian Black Vulture, *Aegypius monachus*, a species of conservation concern

Conservation Genomics: Amalgamation of Conservation Genetics and Ecological and Evolutionary Genomics, ConGenOmics - exchange grant 3922

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*timeframe research exchange:*
22 November 2012 - 19 December 2012
07 January 2013 - 20 January 2013
(6 weeks)

*home institute*:
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## 1. PURPOSE OF THE VISIT

The Eurasian Black Vulture, *Aegypius monachus*, a large raptor species of conservation concern native to southern Europe and central Asia, is subject to serious population declines in the wild. Its wild distribution has shown a dramatic decrease in most of its range and amounts to only less than 2.000 pairs left in Europe (BirdLife International, 2008). As such the species is listed as "near threatened" at world level in the 2008 IUCN Red List and considered even "vulnerable" at European level (BirdLife International, 2008; BirdLife International, 1996). An international conservation breeding program (European Endangered species Programme, EEP), co-ordinated at the Royal Zoological Society of Antwerp (RZSA), and reintroduction projects in southern Europe aim to sustain the captive population in European zoos while also breeding young to be released into the wild, thereby re-establishing wild populations of this species in areas where it has been lost. Sadly, the ambitious program has only minor success with captive breeding - merely a small fraction of captive laid eggs develops into adults (20.6%, 1984-2004, unpublished data Wolfram *et al.*, 2013). This poor reproductive outcome is not a typical feature of the species but is in sharp contrast to high breeding successes reported for wild *A. monachus* (e.g. 69.3%, 2000, Extremadura population, Morán-lópez *et al.*, 2006). Preliminary research and analysis of studbook data suggest poor quality of courtship behaviour and pair bonding to be associated with previous and contemporary failure in captive *A. monachus* breeding

(Pereboom *et al.*, 2005). In a broad scientific approach and collaboration with multiple international partners, the RZSA's Centre for Research and Conservation (CRC) is trying to understand genetics underlying mate choice in *A. monachus*. A special focus is set on the role of major histocompatibility complex (MHC) genes in mate preferences. MHC already has attracted great attention by evolutionary and conservation biologists in the past. A linkage between mate choice or pair bonding and the MHC gene complex, in particular, has been reported for various animals, including rodents (e.g. Yamazaki *et al.*, 1986), birds (e.g. von Schantz *et al.*, 1989; Freeman-Gallant *et al.*, 2003; Ekblom *et al.*, 2004) and primates (e.g. Penn and Potts, 1998; Havlicek and Roberts, 2009), and is believed to be a consequence of the MHC's crucial role in immune responses (Ziegler *et al.*, 2005). Only recently studies characterizing avian MHC, its polymorphism and evolution start addressing the gene complex in birds of prey (Alcaide *et al.*, 2007). The only study addressing MHC in *A. monachus* to date examined exons 1 to 3 of the MHC class II B gene in a single individual from Spain and reported two exon 2 sequences and an absence of stop codons and frameshift mutations in the coding region (Alcaide *et al.*, 2007). There are currently no information available concerning the extend of diversity of exon 2 or the level of gene expression of individual alleles in *A. monachus*, making it impossible to draw conclusions about operating evolutionary mechanisms. In the context of improving captive breeding success and understanding of mate choice criteria in *A. monachus* in this CRC research project, nature and extend of the MHC's involvement in mate preferences is most interesting.

Main purpose of the 6 week research exchange supported by this grant was to genotype a set of Eurasian Black Vultures at their MHC class II B exon 2 loci and a set of mitochondrial markers using massive throughput next generation sequencing (NGS) techniques, namely a GS Junior Titanium platform, with samples (and their known breeding history) provided by the home institute and NGS machines and expertise provided by the host institute. In addition to MHC, sampled individuals were genotyped at two mitochondrial markers (cytochrome *b* and control region) and screened for presence of common blood parasites (per parasite cytochrome *b*) to evaluate general population structure and physical condition.

references:


Alcaide, M., S. Edwards & J. Negro (2007) Characterization, Polymorphism, and Evolution of MHC Class II B Genes in Birds of Prey. *Journal of Molecular Evolution,* **65,** 541-554.

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Ekblom, R., S. A. Saether, M. Grahn, P. Fiske, J. A. Kalas & J. Hoglund (2004) Major histocompatibility complex variation and mate choice in a lekking bird, the great snipe (*Gallinago media*). *Mol Ecol,* **13,** 3821-3828.

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Wolfram, K., Huyghe, M., Galbusera, P., Shephard, J. & Z. Pereboom (2013) *in preparation*

Ziegler, A., H. Kentenich & B. Uchanska-Ziegler (2005) Female choice and the MHC. *Trends Immunol,* **26,** 496-502.

## 2. DESCRIPTION OF THE WORK CARRIED OUT DURING THE VISIT

*Preparations at home institute:*

The research exchange was split into two sections accounting for closure of the host institute from late December 2012 to early January 2013. At the home institute, months prior the research exchange were spent developing an experimental design involving representatives from both institutes and producing PCR products for NGS analysis with a Lib-L chemistry at the host institute. Samples to be ultimately analyzed comprised MHC (2 independent touchdown gradient PCRs) as well as mitochondrial markers in fragments (9 nested PCRs) and markers of plausible accipitrid haemoparasites (2 nested PCRs) for 48 Eurasian Black Vultures and respective negative controls, amounting to a total of 639 samples for analysis. Fragments of interest were barcoded bidirectionally to produce reads in forward as well as in reverse direction. With mate choice being the main interest, the 48 individuals comprised 8 sets of parent(s) and offspring, representing successful breeders, as well as 8 long-term similar-age opposite-sex pairs not yielding offspring, representing unsuccessful breeders. The remaining 13 samples were presumably unrelated individuals with various origins and DNA template quality.

*Processing of samples and library preparation for emPCR amplification at host institute*:

PCR amplification at the home institute underwent massive optimization. At the host institute general success or failure of optimized PCRs was to be tested for few representative sample and marker combinations by means of gelelectrophoresis (1.5% agarose gels with SYBR-stained samples, 75 min at 120 V; mtDNA markers). Gelelectrophoresis of MHC markers, despite massive efforts for optimization, indicated persistent presence of low size fragments (i.e. unspecific products, primer-dimer artifacts) able to interfere in further analysis and requiring purification of MHC amplicons. MHC fragments of interest were therefore extracted from 1.5% agarose gels (SYBR-stained samples, 75 min at 100 V) separately for each individual and PCR with the Qiaex II gel extraction kit, purified according to the manufacturer and stored in 0.5x TE buffer. Concentration of each purified MHC extract as well as each unpurified PCR product of mitochondrial markers was determined with PicoGreen dsDNA quantification according to the manufacturer and in non replicated measurement. Samples were then normalized with 1x TE buffer and pooled to $10^6$ molecules per µL for further use in emPCR amplification or titration. During the research exchange three runs of a GS Junior Titanium were carried out (14 Dec 2012, 09 Jan 2013 and 18 Jan 2013). In the first run, MHC amplicons and mitochondrial amplicons contributed equally to the final pool subjected to NGS, whereas MHC amplicons were subjected to the second and third run in solo.

*emPCR, titration, emPCR and initial run* (14 Dec 2012):

An initial emPCR using 0.5 copies of amplicon per enrichment bead was carried out according to the manufacturer's protocol. Over-enrichment of beads, unwanted presence of multiple amplicons per bead, became evident in the course of the emPCR and prompted titration. Titration, i.e. parallel emPCR at 0.5, 0.2, 0.1 and 0.01 copies per bead, indicated optimal enrichment to occur below the minimum concentration of 0.01 copies per bead. This observation was later attributed to artifacts from mitochondrial marker PCRs that were, in contrast to MHC artifacts, not removed prior to emPCR. The initial run was eventually carried out with < 0.01 copies per bead by proceeding with of only a fraction of emPCR enriched beads. The run provided sequences for most mitochondrial marker fragments and individuals, but data for MHC did not suffice in number of reads (see below). A second GS Junior Titanium run was launched.

*titration, emPCR and second run* (09 Jan 2013):
With mate choice and MHC being the main interests, the second run was performed on MHC amplicons exclusively. A titration was done employing pooled MHC amplicons at 2.0, 0.5, 0.1 and 0.01 copies per bead. The second run was eventually performed with approximately 0.04 copies per bead. Raw data generated in the second run again indicated results of insufficient quality (see below), which staff at the host institute related to technical problems of the GS Junior Titanium platform.

*repeat of second run* (18 Jan 2013):
Following reprocessing of GS Junior Titanium raw data and troubleshooting with the platform's manufacturer, the second run was repeated once more for MHC amplicons exclusively at the very end of the research exchange.

Most lab work at the host institute was done under supervision by, assisted by or, as in case of emPCRs and GS Junior Titanium runs, by local lab manager Dipl. Biol. S. Mbedi.

*data analysis:*
Raw data were initially inspected with the GS Junior Titanium run browser for number of library reads, read lengths and percentage of filter passage. Raw data were then transferred to the host institute's server, passed through sets of filters and subjected to Geneious R6.0.5. In this software reads were divided according to MID barcodes and written into a fasta-file per individual vulture. The Linux command routine in Table 1 was then used to extract sequences for each marker fragment per individual vulture.

Table 1: Data processing routine for mitochondrial markers in Ubuntu 12.10 terminal.

| **target** | **command** | **action** |
|---|---|---|
| MID | makeblastdb | creating new blast database for all mitochondrial markers of current MID |
| **cytochrome *b*** | blastn | searching above nucleotide database for total cyt *b* reference |
| | awk | retrieving read names matching reference |
| | fnafile | constructing fasta file with reads matching reference |
| | makeblastdb | creating new blast database from above fasta file for cyt *b* of current MID |
| fragment 1 - 5 | blastn | searching above database for exclusive sequences of each fragment |
| fragment 1 - 5 | awk | retrieving read names matching exclusive sequences |
| fragment 1 - 5 | fnafile | constructing fasta file with reads matching exclusive sequences |
| **control region** | blastn | searching top nucleotide database for total control region reference |
| | awk | retrieving read names matching reference |
| | fnafile | constructing fasta file with reads matching reference |
| | makeblastdb | creating new blast database from above fasta file for control region of current MID |
| fragment 1, 3, 4 | blastn | searching above database for exclusive sequences of each fragment |
| fragment 1, 3, 4 | awk | retrieving read names matching exclusive sequences |
| fragment 2 | cat | concatenating exclusive sequences for fragments 1, 3, 4 to define fragment 2 |
| fragment 1 - 4 | fnafile | constructing fasta file with reads matching exclusive sequences |
| **parasite 1** cyt *b* | blastn | searching top nucleotide database for total parasite 1 and 2 cytb *b* reference |
| and | awk | retrieving read names matching reference |
| **parasite 2** cyt *b* | fnafile | constructing fasta file with reads matching reference |
| | tblastx | searching top nucleotide database for parasite cytb *b* references using translations in all possible reading frames |
| | awk | retrieving read names matching reference |
| | fnafile | constructing fasta file with reads matching reference |

Further manipulation and processing of sequences (i.e. trimming, editing, assembling, aligning, annotating) was then again performed in Geneious on fasta-files.

Data analysis at the host institute was introduced and initially supervised by Dr. C. Mazzoni.

## 3. DESCRIPTION OF THE MAIN RESULTS OBTAINED

As expected from a NGS approach, a great amount of data had been collected in the GS Junior Titanium runs. The following present results available at the end of the research exchange and is hence an overview of raw and basic semi-processed data available for subsequent further analysis.

*mitochondrial markers*

For most individuals and mitochondrial markers, mitochondrial sequences were successfully recovered. For recovery of some individuals, notably the same individuals in the various markers, data filters had to be adjusted to incorporate problematic template DNA or MID identifiers into the analysis. Table 2 summarizes the amount of data collected for mitochondrial markers with initial filtering (further MIDs were recovered with modified filtering at a later stage). Even considering that bidirectional sequencing was performed, Table 2 indicates that a NGS as in the present experimental design is producing a vast excess of data for a mitochondrial marker compared to traditional Sanger sequencing. Taking into account time and cost efficiency, the latter technique appears to suffice for mitochondrial markers of interest in this project.

Table 2: Overview of mitochondrial data obtained in the initial GS Junior Titanium run (14 Dec 2012). Number of MIDs corresponds to number of individuals (out of 48 vultures plus 2 blanks) for which sequences were recovered. Number of total sequences shows total number of reads for all individuals obtained for each marker fragment.

| marker | # MIDs (of 50) | # total sequences | read length range | expected length |
|---|---|---|---|---|
| **cytochrome *b*** | | | | |
| fragment 1 | 33 | 8530 | 197 - 1170 bp | 414 bp |
| fragment 2 | 33 | 8167 | 359 - 1160 bp | 311 bp |
| fragment 3 | 33 | 8145 | 221 - 1169 bp | 314 bp |
| fragment 4 | 35 | 17998 | 301 - 970 bp | 291 bp |
| fragment 5 | 35 | 8066 | 360 - 1168 bp | 354 bp |
| **control region** | | | | |
| fragment 1 | 35 | 13897 | 254 - 1170 bp | 392 bp |
| fragment 2 | 35 | 24341 | 75 - 1169 bp | 209 bp |
| fragment 3 | 34 | 6973 | 168 - 1147 bp | 343 bp |
| fragment 4 | 34 | 17532 | 75 - 1161 bp | 208 bp |
| **parasite cyt *b* 1** | | | | |
| tblastx | 35 | 27339 | 124 - 1170 bp | unknown |
| blastn | 1 | 3 | 720 - 843 bp | unknown |
| **parasite cyt *b* 2** | | | | |
| tblastx | 35 | 33660 | 124 - 1170 bp | unknown |
| blastn | 1 | 3 | 720 - 843 bp | unknown |

*MHC*

The initial GS Junior Titanium run (14 Dec 2012) indicated underrepresentation of MHC when concurrently sequenced with mitochondrial markers. Consequently, this run produced MHC data that suffice only in case

of a few individuals to determine number and sequence of MHC class II B exon 2 (see Figure 1 and Figure 2). For these few individuals, presence of at least 4 to 6 MHC alleles per individual is suggested, arguing for possibly 3 or more respective loci. This finding is in agreement with several related Griffon vulture species and was not described for this very species before (see Alcaide *et al.*, 2007).

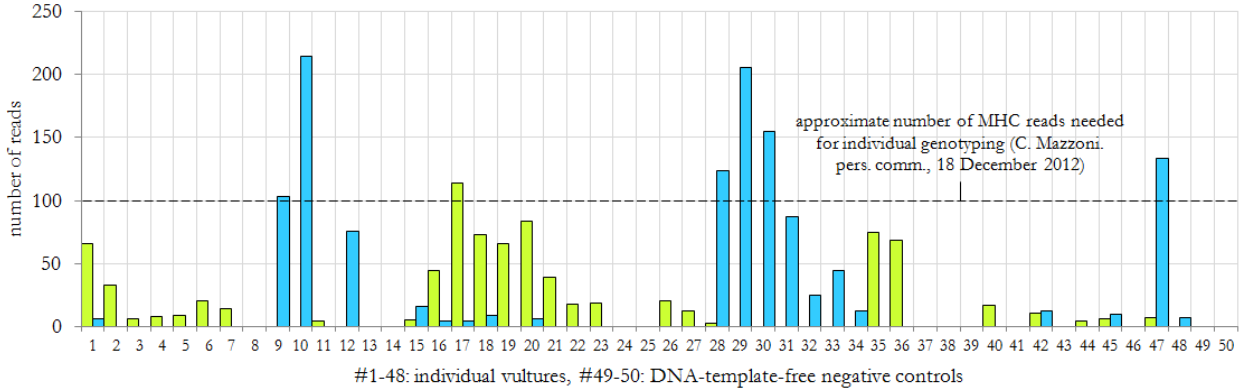


Figure 1: Number of reads for MHC class II B exon 2 obtained in initial GS Junior Titanium run in 48 Eurasian Black Vultures (in replicate, green: original PCR, blue: replicate PCR) and two negative controls as retrieved with initial filter settings.


Figure 2: Data collected for vulture individual 19 in first GS Junior Titanium run indicating existence of multiple MHC class II B exon 2 loci in this species. Out of 476 total reads for this individual (filter settings different to Figure 1), sequences 1 to 6 represent sequences present in 10 or more reads (number of identical reads given in parentheses, see arrow), all 6 translating into a separate amino acid sequence. Sequences 7 to 13 (pale) are also present in 10 or more reads for this individual, however, translate into a premature stop codon. A presence of 6 or more alleles argues for at least 3, and possibly up to 6, MHC loci in *A. monachus*.

The second GS Junior Titanium run (09 Jan 2013), for MHC amplicons exclusively, again did not produce satisfying results. Particularly, quality of sequence reads beyond 100 bp of the expected approximately 330 bp was insufficient in this run, which was attributed to technical problems (C. Mazzoni pers. comm., 17 Jan 2013). Modification of data processing routine, reprocessing of raw data and troubleshooting with the manufacturer allowed for little improvement. The run for MHC amplicons was repeated for a third time (18 Jan 2013). Results of the latest run were not available by the end of the 6-week exchange period or submission of this report and are yet to be analyzed.

In conclusion, NGS technology on a GS Junior Titanium platform appears not suitable for a complex experimental design as in the present case. Concurrent analysis of MHC and multiple mitochondrial sequences, even with as much as half of the platform being designated for MHC analysis, failed producing sufficient number and quality of MHC reads while mitochondrial reads are profuse and excessive. As a consequence, future employment of this technology will be focused on MHC analysis alone.

reference:

Alcaide, M., S. Edwards & J. Negro (2007) Characterization, Polymorphism, and Evolution of MHC Class II B Genes in Birds of Prey. *Journal of Molecular Evolution,* **65,** 541-554.

## 4. FUTURE COLLABORATION WITH HOST INSTITUTION (IF APPLICABLE)

An analysis of further samples is intended at a future point in collaboration between the CRC and BeGenDiv.

## 5. PROJECTED PUBLICATIONS/ARTICLES RESULTING OR TO RESULT FROM THE GRANT (ESF must be acknowledged in publications resulting from the grantee's work in relation with the grant)

*in preparation*:

Wolfram, K., Galbusera, P., Huyghe, M., Shephard, J., Mazzoni, C., Pereboom, Z., Matthysen, E. 2013 MHC in the Eurasian Black Vulture *Aegypius monachus* and its relevance in mate choice and captive breeding in a species of conservation concern (working title)

## 6. OTHER COMMENTS (IF ANY)

I thank the committee for allowing to postpone my exchange visit twice in 2012, allowing me to further optimize initial PCR conditions before proceeding to the host institute. These additional optimizations significantly contributed to a better amplification.