HOGESCHOOL UTRECHT

Ravenwerkgroep Nederland *krrok*

Sex determination of nestling ravens (*Corvus corax*) by PCR genotyping
Correlation with biometric parameters and application in behavioral studies

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The population size of the common raven (*Corvus corax*) in the Netherlands steadily increased over the last decades, thanks to an active reintroduction program. Research into distribution, mating- and feeding behavior of these birds is performed by volunteers from 'Ravenwerkgroep Nederland', who monitor nests and track individual birds by ringing and transmitter mounting. These studies are impaired by the fact that the raven is a monomorphic species, implying that phenotypic sexing of living birds is virtually impossible.

Students and staff from Hogeschool Utrecht implemented a PCR-based genotyping assay, based on intron-length variation in the sex chromosome-located CHD1 gene. DNA isolated from a single neck feather suffices to distinguish males (ZZ) from females (ZW).

Possible correlations between PCR-established sex and (combinations of) various biometric parameters were statistically explored using a dataset obtained from 101 individual birds, employing a decision tree approach (Random Forest model). Despite the raven's highly monomorphic phenotype an accuracy of 0.75 was reached (after correction for age).

FIELDWORK

When observing the common raven it is virtually impossible to distinguish males from females, neither as chicks nor as adults.[1,2,3] (**A**, **B**)

Neck feathers from raven chicks were collected for genotyping. (**B**, **C**)

Isolated chicks were ringed and various biometric parameters were assessed, including weight, wingspan, leg thickness and claw size before replacement in their nest. (**C**)

In several cases GPS transmitters were mounted, enabling flight path tracking and territory mapping. (**D**, **E**)

Phenotypic parameter	Males*		Females*		p-value
Wingspan (mm)	243.5 ± 50.9	(n=47)	243.8 ± 46.8	(n=54)	0.98
Age (days)**	32.2 ± 6.1	(n=47)	32.2 ± 5.4	(n=54)	1.00
Weight (gr)	997.3 ± 160.4	(n=47)	933.6 ± 102.2	(n=54)	0.02
Claw size (mm)	110.5 ± 6.2	(n=47)	105.4 ± 5.7	(n=54)	<0.01
Leg thickness (mm)	10.22 ± 0.58	(n=47)	10.0 ± 0.6	(n=54)	0.07

*Determined by PCR, **Deduced from wingspan

STATISTICS

Phenotypic parameters of 101 individual birds were divided into a male and a female group and average values ± SD were calculated.

Despite the alleged morphologic similarity between sexes, significant differences were observed in average body weight (females lighter) and claw size (females smaller), as established with a two-sided unpaired T-test. This confirms a previous study (Bedrosian *et al*, 2008).

To establish a statistical prediction model for sex estimation, we trained a random forest machine learning model on the morphometric data set. To establish an unsupervised exploratory analysis of the data we first explored the segregation of sex using all predictors with Factor Analysis of Mixed Data. This showed an overall good segregation of the sex. After removing age as a predictor, because it is highly correlated with other predictors and cannot be determined with high confidence in wild ravens, we then moved on to determining variable importance using a decision tree approach. After splitting the data into a training and a test set, we trained a Random Forest model for binary classification on sex being either male or female[4,5] The overall accuracy on the test set (30% of the data) of this model with 79 unique ravens was 0.75. The area under the ROC was 0.792.

To accommodate wildlife observers in using this prediction model in the field, the model was implemented in a Shiny app, where observers can enter raven attributes and get a sex prediction of the animal on the spot[6]. The Shiny app also outputs the model's class probability to accommodate for uncertainty of the prediction.

For further information on R and Shiny: https://rstudio-connect.hu.nl/raven-app and marc.teunis@hu.nl

GENOTYPING

Genomic DNA was isolated from scalpel-sliced and digested (proteinase K, SDS and DTT) calamuses by ammonium acetate and isopropanol, and subsequent ethanol-precipitation.

Male ravens carry ZZ sex chromosomes and females WZ. Both W and Z chromosomes carry the CHD1 (Chromo Helicase DNA-binding) gene. (**F**)

W and Z-dependent intron-length variation occurs within the CHD1 gene, which provides a target for genetic sex determination.[1] *Corvus corax* genome sequence information was not available, therefore CHD1 sequence of the closely related *Corvus cornix* was used to design primers (→) flanking CHD1 intron 17 (forward: CTACTGATTCGTCTGCGAGA, reverse: ATTAAAATGATCCAGTGCTTGT). (**G**)

Among bird species CHD1 intron 17 PCR amplicon length varies 150 to 250 bp between W and Z alleles, enabling discrimination between ZZ (♂) and WZ (♀) chromosome sets by agarose gel electrophoresis.[2,3] (**H**)

Sex was determined of 101 raven chicks of which an example gel is shown below. DNA from male and female kestrel blood samples served as controls, since sexes are phenotypically distinguishable in this species.

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