1   # Automatic identification of bird females using egg phenotype

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12

13   **Abstract**

14   1. Identification of individuals greatly contributes to understanding animal ecology and

15   evolution, and in many cases can only be achieved using expensive and invasive

16   techniques. Advances in computing technology offer alternative cost-effective techniques

17   which are less invasive and can discriminate between individuals based on visual and/or

18   acoustic cues. Here, we employ human assessment and an automatic analytical approach

19   to predict the identity of common cuckoo (*Cuculus canorus*) females based on the

20   appearance of their eggs. The cuckoo's secretive brood parasitic strategy makes studying

21   its life history very challenging. Eggs were analysed using calibrated digital photography

22   for quantifying spotting pattern, size and shape, and spectrometry for measuring colour.

23   Cuckoo females were identified from genetic sampling of their nestlings, allowing us to

24   determine the accuracy of human and automatic female assignment. Finally, we used a

25   novel 'same-different' approach that uses both genetic and phenotypic information to

26   assign eggs that were not genetically analysed.

27   2. Our results supported the 'constant egg-type hypothesis', showing that individual cuckoo

28   females lay eggs with a relatively constant appearance and that eggs laid by different

29   females differ more than eggs laid by the same female. The accuracy of unsupervised

30   hierarchical clustering was comparable to assessments of experienced human observers.

31   Supervised random forest analysis showed better results, with higher cluster accuracy.

32   Same-different analysis was able to assign 22 of 87 unidentified cuckoo eggs to seven

33   already known females.

34   3. Our study showed that egg appearance on its own is not sufficient for identification of

35   individual cuckoo females. We therefore advocate genetic analysis to be used for this

36   purpose. However, supervised analytical methods reliably assigned a relatively high

37   number of eggs without genetic data to their mothers which can be used in conjunction

38   with genetic testing as a cost-effective method for increasing sample sizes for eggs where

39   genetic samples could not be obtained.

40

41

42   **Introduction**

43   Identification of individuals is important in zoological research, particularly when investigating

44   variation among or within individuals in a population. Traditionally, capture-mark-recapture

45   techniques have been used to monitor individuals during their lifetime (Lindberg, 2012; Jung,

46   Boonstra, & Krebs, 2020). This method has been improved by employing more sophisticated

47   methods such as attaching GPS (global positioning system) and radio transmitters or RFID (radio

48   frequency identification) tags (Krause et al., 2013) that allow researchers to investigate the spatial

49   activity of animals in more detail. However, these methods still require capturing and tagging that

50   is usually time-consuming, expensive (depending on the method used), and may reduce animal

51   welfare (Weinstein, 2018). Therefore, cost-effective indirect approaches have been developed to

52   identify and monitor individuals within the same species.

53

54 These indirect approaches rely on the fact that individuals differ from each other visually or

55 acoustically and this variation may be used for their identification. Indeed, it has been shown that

56 e.g. face (Deb et al., 2018; Hansen et al., 2018; Hou et al., 2020) and body pattern data (Hiby et

57 al., 2009; Bolger, Morrison, Vance, Lee, & Farid, 2012; Crall, Stewart, Berger-Wolf, Rubenstein,

58 & Sundaresan, 2013; Ferreira et al., 2020) captured from photographs may allow discrimination

59 of individuals. Similarly, sounds produced by animals (especially bird song) also seem to serve

60 as a good individual fingerprint (Blumstein et al., 2011; Petrusková, Pišvejcová, Kinštová, Brinke,

61 & Petrusek, 2016; Ptacek, Machlica, Linhart, Jaska, & Muller, 2016). Recently, applying modern

62 computer technology and artificial intelligence techniques (such as convolutional neural networks)

63 that automate the analysis of various types of data from different sources such as pictures or

64 audio recordings has made these methods reliable and applicable for various animal taxa

65 (Hansen et al., 2018; Christin et al., 2019; Ferreira et al., 2020; Hou et al., 2020).

66

67 However, for many species, identification of all individuals in a population is still not

68 straightforward e.g. due to their secretive behaviour or due to the fact that it is difficult to catch

69 them. Here, we focus on one group of animals that are especially challenging to study – avian

70 brood parasites. There are more than a hundred obligate brood parasites that never build their

71 own nests and instead lay their eggs into nests of other species (Davies, 2010). Even more

72 species belong to conspecific brood parasites that only occasionally lay eggs into nests of the

73 unrelated conspecifics (Bruce E. Lyon & Eadie, 2008). Brood parasites and their hosts have been

74 the focus of considerable research into co-evolutionary arms races (Soler, 2017). But since brood

75 parasites only lay eggs and then usually do not return to host nests (but see Šulc et al. 2020),

76 and because egg laying is (especially in obligate brood parasites) very quick (McMaster, Neudorf,

77 Sealy, & Pitcher, 2004; Gloag, Fiorini, Reboreda, & Kacelnik, 2013; Jelínek, Šulc, Štetková, &

78 Honza, 2020), direct observation of parasitism in nature is difficult and identification of parasitic

79   females is problematic. As a consequence, many important aspects of females' life history

80   strategy are still poorly understood; in obligate brood parasites, we e.g. still know little about

81   spatio-temporal distribution of their egg laying, consistency in host selection and the total number

82   of eggs they lay during a breeding season. Conspecific brood parasitism is even less understood

83   and we do not even know why some females adopt this strategy (Bruce E. Lyon & Eadie, 2008).

84

85   The idea of identifying bird females according to the appearance of the eggs they laid depends

86   on the presumption that within-clutch variation in egg appearance is lower than between-clutch

87   variation which has been confirmed for several species including brood parasites (Øien, Moksnes,

88   & Røskaft, 1995; McRae & Burke, 1996; Paillisson, Latraube, Marion, & Bretagnolle, 2008; Höltje,

89   Mewes, Haase, & Ornés, 2016). This approach has therefore been applied (although with

90   caveats) for the identification of parasitic eggs in some conspecific brood parasite species

91   (Gibbons, 1986; Møller, 1987; Jackson, 1992; Petersen, 1992; Bruce E. Lyon, 1993; McRae &

92   Burke, 1996; B. E Lyon, 2003). However, some studies that estimated accuracy of parasitic egg

93   identification showed ambiguous results for some species (Ådahl, Lindström, Ruxton, Arnold, &

94   Begg, 2004; Pöysä, Lindblom, Rutila, & Sorjonen, 2009; Eadie, Smith, Zadworny, Kühnlein, &

95   Cheng, 2010; Lemons, Sedinger, & Randle, 2011; Petrželková, Pöysä, Klvaňa, Albrecht, & Hořák,

96   2017) and for others this method did not work at all (Brown & Sherman, 1989; Cariello, Lima,

97   Schwabl, & Macedo, 2004; Grønstøl, Blomqvist, & Wagner, 2006; Griffith, Barr, Sheldon, Rowe,

98   & Burke, 2009; Roy, Parker, & Gates, 2009). One of the reasons why many studies found low

99   accuracy of identification might be that closely related females lay indistinctive eggs. Several

100   studies showed that egg appearance, namely egg color (Wei, Bitgood, & Dentine, 1992; Collias,

101   1993; Morales et al., 2010), spotting pattern (Gosler, Barnett, & James Reynolds, 2000) and egg

102   size (Christians, 2002) are highly heritable traits which might complicate such analyses especially

103   in inbred populations. Another explanation might be that previous studies did not use the full

104   potential of egg variability (e.g. none of the presented studies measured egg colour in the

105   ultraviolet range of spectrum).

106

107   Identification of parasitic females using egg appearance has also been attempted in the common

108   cuckoo (hereafter cuckoo), but was unsuccessful (Moksnes et al., 2008). However, the study

109   assessed cuckoo eggs from a human perspective, with people sorting the eggs based on

110   photographs. To date, there have been no attempts to use more objective quantification methods

111   for egg classification in the cuckoo. These objective methods, such as spectrophotometry for

112   measuring colours (including the ultraviolet part of the spectrum), and image analysis of

113   photographs for quantifying spotting pattern, size and shape of eggs are now available, and may

114   allow more accurate classification that can be carried out in an automated manner.

115

116   In this study, we employ a novel automatic analytical approach to analyse phenotypic features of

117   cuckoo eggs such as dimensions (size, shape), colour and spotting pattern to predict maternal

118   identity. If successful, this low-cost and minimally invasive female identification method would

119   greatly facilitate studies into a range of key questions regarding this secretive brood parasitic

120   species. We also performed human assessment based on sorting of photos with cuckoo eggs to

121   compare the reliability of both methods with the true identity acquired from molecular analyses.

122   Moreover, we believe this automatic technique might be also used in other brood parasitic

123   systems or in species where females are difficult to catch (see e.g. Höltje et al. 2016). Finally, it

124   has been suggested that similarly looking eggs laid by different cuckoo females may belong to

125   closely related females, e.g. mother and daughter (Moksnes et al., 2008). Therefore, we will for

126   the first time investigate the relationship between the genetic distance of individual cuckoo

127   females and the phenotypic distance of their eggs.

128

129

130 **Material and Methods**

131 *Study system and data collection*

132 All data were collected in the fishpond area between Mutěnice (48°54′N, 17°02′E) and Hodonín

133 (48°51′N, 17°07′E) in South Moravia, Czech Republic from May to July 2017. We systematically

134 searched the littoral vegetation for the great reed warbler (*Acrocephalus arundinaceus*) and

135 Eurasian reed warbler (*Acrocephalus scirpaceus*) nests. Most great reed warbler (hereafter GRW)

136 nests were found during the building stage when mapping male territories and mating status

137 (Bensch, 1996). The rest of the GRW and all Eurasian reed warbler (hereafter RW) nests were

138 found in different stages of breeding by systematic searching. If possible, all GRW nests were

139 checked every day from the nest building stage until clutch completion and approximately every

140 third day during incubation. All RW nests were checked approximately every second day during

141 laying stage and extensively during incubation. GRWs experienced 92 % (59 out of 64 nests) and

142 RWs 20 % (91 out of 456 nests) cuckoo parasitism rate. Multiple parasitism was also common;

143 37 of 59 and two of 91 parasitized GRW and RW nests, respectively, were parasitized by more

144 than one cuckoo egg.

145

146 When a cuckoo egg was found in a host nest, we immediately measured its colour and took a

147 photo (see below) to avoid colour change during the incubation period (Hanley et al., 2016). In

148 the cases of multiply parasitized nests, we removed the newly laid cuckoo egg(s), transferred

149 them to an incubator (HEKA-Kongo; HEKA-Brutgeräte, Rietberg, Germany) and incubated them

150 artificially to prevent sample losses caused by the cuckoo nestlings (Honza, Vošlajerová, &

151 Moskát, 2007). The removed cuckoo eggs were either incubated until hatching and chicks placed

152 into non-parasitized host nests (for other experiments) or we froze the eggs before hatching for

153 the future genetic analysis (see *Genotyping and kinship analysis* section). We took a blood

154 sample from all 10-days old cuckoo nestlings from their ulnar or medial tarsometatarsal vein

155 (approx. 25 μl). Finally, we mist-netted 29 and 16 adult cuckoo males and females, respectively,

156 and collected their blood samples from the ulnar vein (approx. 25 µl). All blood samples were

157 stored in 96% ethanol until later genetic analyses.

158

159 Altogether we found 203 cuckoo eggs (121 and 82 in the GRW and RW nests, respectively). We

160 photographed and measured the colour of 192 of them. Among these photographed cuckoo eggs,

161 genetic samples were collected from 105 nestlings or embryos.

162

163 *Measurements of egg appearance*

164 To obtain background colour we measured reflectance using JAZ Spectrometer (Ocean Optics,

165 Dunedin, FL, U.S.A.) in the range 300–700 nm, as that is the wavelengths range birds can

166 perceive (Cuthill 2006). We took nine measurements (each covering approximately 1 mm^2) at

167 three different parts of the egg (sharp pole, middle part and blunt pole). Since we focused on

168 background colour, we avoided measuring dark spots (Šulc et al. 2016). For each egg, we used

169 the measurement with the highest reflectance that best corresponded to the colour of the

170 background (Šulc et al. 2019).

171

172 Spotting pattern was calculated from digital images taken by a Canon EOS 700D with prime

173 Canon EF 40 mm lens. All photos were taken under standardized diffuse sunlight conditions

174 (using a photography light tent), at the same angle and from the same distance and were referred

175 to a grey standard (X-Rite Colour Checker Grey Scale Chart) with known reflectance. Image

176 calibration, pattern analysis, analysis of shape and measurements of size were performed in

177 ImageJ software (Schneider et al. 2012) using the Multispectral Image Calibration and Analysis

178 (MICA) Toolbox (van den Berg et al. 2020). A scale bar was included in each photo, meaning that

179 all images were equally rescaled to the scale of the smallest image (30 pixels/mm), and egg

180 dimensions were obtained from the photos. For pattern investigation we applied a granularity

181 analysis approach (Troscianko and Stevens 2015) that creates a bandpass 'energy' spectrum

182   across a range of spatial frequencies, and then the pattern energy at each frequency band was

183   measured as the standard deviation of the filtered image (for details see Šulc et al. 2019 and van

184   den Berg et al. 2020). Since pattern energy does not discriminate the direction of the pattern (it

185   cannot distinguish between dark spots on light background and light spots on dark background),

186   we also calculated the 'skew' of the pattern, which quantifies the asymmetry of the pattern

187   luminance distribution. A negative value of skew implies there are more spots than background

188   colour, while a positive skew implies there is more background colour than spots. Skew was

189   measured independently at each granularity band. All colour measurements and photos were

190   taken by a single person (M.Š.) to ensure high consistency of the data.

191

192   *Genotyping and kinship analysis*

193   DNA was isolated from the blood of adults and nestlings or tissues of embryos. (65 nestlings, 41

194   embryos, 29 adult males and 16 adult females). We estimated kinship relationships from nuclear

195   SNPs and mitochondrial DNA haplotypes enabling us to exclude highly implausible maternal (or

196   maternal-sibling) relationships in the inferred genealogy. As an additional criterion we also used

197   the laying date of cuckoo eggs (data from daily checks) because it is known that a cuckoo female

198   cannot lay her eggs more often that every second day (Chance, 1922; Payne, 1973; Wyllie, 1981).

199

200   To acquire the SNP dataset, we genotyped all samples with the ddRAD (double digest restriction-

201   site associated DNA) technique (Petersen, 1992) following the protocol of (Piálek et al., 2019).

202   Two prepared libraries were sequenced on an Illumina HiSeq4000 system (2 lanes, 150 cycles

203   P/E) in the EMBL Genomic Core Facility, Heidelberg, Germany. The obtained RAD-tags were

204   processed in Stacks v2.4 (Catchen et al. 2011, Rochette et al. 2019) and mapped on the Cuculus

205   canorus genome GCA000709325.1 (https://www.ncbi.nlm.nih.gov) with Bowtie2 assembler

206   v2.2.4 (Langmead & Salzberg, 2012). Only loci with 95% or higher presence of individuals were

207   scored and further filtered based on Hardy–Weinberg equilibrium, linkage disequilibrium and

208   minimum minor allele frequency (0.4) in PLINK v1.9 (Purcell et al., 2007) which resulted in a

209   dataset with 1620 markers. Kinship relationships were estimated using Colony (Jones & Wang,

210   2010) based on >1000 nuclear SNPs.

211

212   For the mitochondrial haplotype analysis, we sequenced a 411-bp portion of the left-hand

213   hypervariable control region (Gibbs et al., 2000; Fossøy et al., 2011, 2012). Mitochondrial

214   sequence data were assembled and manually checked in Geneious v10.2.6 (Kearse et al., 2012)

215   and haplotypes were estimated based on a distance matrix with up to 1% tolerance (approx. 4

216   mutations) for genotyping errors.

217

218   Kinship analysis assigned the offspring (n = 105) to 30 clusters containing 1–10 eggs each (Fig.

219   1–5 in Supplementary material). Among these 30 clusters, nine corresponded to females that

220   were caught and genotyped as described above. Thus, we were able to calculate genetic

221   distances among these females.

222

223   For subsequent analysis dealing with egg phenotype (human and automatic assessment, see

224   below), we removed females to which only one egg has been assigned (n = 10), meaning that we

225   used a final dataset of 95 eggs laid by 20 females.

226

227   *Human assessment*

228   We printed 95 photographs of cuckoo eggs that were standardized in their colour and size using

229   the MICA Toolbox (van den Berg et al. 2020; Fig.1–7 in supplementary material). We then asked

230   twelve people to sort these photographs and create groups of pictures representing individual

231   females according to similarity in egg appearance. Firstly, we asked them to sort these pictures

232   into an unknown number of groups and, secondly, we asked them to sort these pictures into 20

233   groups corresponding to the real number of females identified by genetic assessment of identity.

234   For the assessments, we asked 1) five people with no experience with egg appearance from wild

235   animals, 2) three students of avian ecology that had experience with egg appearance from wild

236   birds but had never seen cuckoo eggs and 3) four people (mostly co-authors of this manuscript)

237   that had years of experience with cuckoo eggs. All participants received no other information

238   about the eggs. Cluster similarity in egg classification was assessed using the adjusted Rand

239   index, which provides a corrected-for-chance measure of the similarity between two data

240   clusterings, implemented using the 'cluster_similarity' function from the R package clustereval

241   (Ramey, 2012).

242

243   *Automatic assessment*

244   We developed an automatic method based on the similarities/differences of cuckoo egg

245   phenotypes. In the first step, we collected colour, pattern and dimension data from calibrated

246   photographs and spectrophotometry data (for details, see Šulc et al., 2019) for all cuckoo eggs.

247   Initially, we conducted Principal component analysis (PCA) on different aspects of the egg

248   photographs, in order to avoid the use of correlated variables in the models.

249

250   *Spectral data:* a spectrophotometer was used to assess the background colour of cuckoo eggs

251   (for details, see *Measurements of egg appearance*). PCA was carried out using binned, scaled

252   spectral data created in the R package *pavo* (Maia, Gruson, Endler, & White, 2019), and two

253   spectral PCA components were used in the final dataset (based on scree plot inspection). We

254   also used two other spectral measures extracted from *pavo*: the mean brightness (B2; mean

255   relative reflectance over the entire spectral range) (Hill & Mcgraw, 2006) and the position of the

256   ultraviolet (UV) peak (defined as a wavelength within the range of 300–360nm where reflectance

257   reached the highest point).

258

259   *Shape data:* the variables entered into the PCA were length, maximum width, volume, ellipse

260   deviation and surface area (Troscianko, 2014). Three shape PCA components were selected for

261   inclusion into the final dataset based on scree plot inspection.

262

263   *Pattern data:* the variables entered into the PCA were 12 pattern energies measured at a range

264   of scales (from 1 to 0.0221 in steps of 1/square root of 2) across the whole egg (Troscianko &

265   Stevens, 2015), and 12 pattern energy skew values measured at the same range of scales across

266   the whole egg. We also included a measure of total pattern energy across the whole egg. Finally,

267   we divided up each egg into three segments and measured the total pattern energy in each

268   segment as well as the standard deviation between segments, to get a measure of how variable

269   the patterning was across the egg. Three pattern PCA components were selected for inclusion

270   into the final dataset based on scree plot inspection.

271

272   *Luminance data:* we analysed luminance from photographs, including both the spots and

273   background areas of the eggs. We divided the egg up into three segments and took the average

274   luminance and the standard deviation of luminance across each segment, as well as the standard

275   deviation of luminance across all three segments. One luminance PCA component was selected

276   for inclusion into the final dataset based on scree plot inspection.

277

278   In total, the final dataset contained 11 egg phenotypic traits that were used for clustering analysis.

279

280   *Within- and between-female variability in egg appearance*

281   To create a metric of within-female variance, we calculated the standard deviation for each

282   phenotypic trait within one female, and then took a mean value across all traits, giving an average

283   variability value for each female.

284   To create a metric of between-female variance, we calculated the average value of each

285   phenotypic trait for each female (i.e. created an "average" egg) and then calculated the standard

286   deviation for each phenotypic trait across all females. We then averaged these standard

287   deviations to create a measure of between-female variability across all traits. All trait values were

288   scaled to ensure comparability across different traits.

289   To test whether within-female variance is lower than between-female variance, we conducted a

290   one-sample t-test where the within-female variance metric (n=20) is compared with the test value

291   (the between-female variance value).

292

293   *Unsupervised learning*

294   Firstly, we carried out hierarchical clustering to attempt to cluster the eggs via visual similarity

295   without any training or further information (e.g. number of females present). All variables were

296   scaled for this analysis. To assess the accuracy of this method, we cut the tree by specifying the

297   real number of groups (20) and assessed the cluster similarity between the predictions of the

298   hierarchical model compared to the real data using the adjusted Rand index, as before.

299

300   *Supervised learning*

301   *Female clustering*: We used a random forest model with a 'leave-one-out' cross-validation

302   approach (Stone, 1974). For each egg in the dataset, the model was trained using a dataset

303   consisting of all other eggs, and then tested using the focal egg. The model attempted to classify

304   each egg to a given female, and the accuracy of the model was assessed using the classification

305   accuracy value, and through cluster similarity values, as before (taking the average of 1000 runs,

306   as random forest modelling is a stochastic process). We also fitted a random forest model to the

307   full dataset to allow us to assess the importance of the different variables included in the model

308   (using the mean decrease in accuracy).

309

310   *Same/different analysis*: We used an approach where a random forest model was trained to label

311   pairs of eggs as 'same' or 'different'. The training set used 3000 'same' rows, where the two eggs

312   were from the same female (but are not identical to each other) and 3000 'different' rows, where

313   the two eggs were from different females.

314

315   To test our models, we tested each egg in the labelled dataset on all eggs sequentially, including

316   itself. We first tested whether the model recognised the identical eggs as being the same. We

317   then tested whether each egg was only paired with other eggs from the same female i.e. whether

318   the model could uniquely identify clusters of eggs that belonged together. The entire process

319   (creating a training set, training the random forest model and testing the model) was repeated

320   1000 times.

321

322   For the unlabelled dataset, we calculated how many times in each of these 1000 runs the target

323   egg was matched with a cluster of eggs that were from the same female. If the percentage was

324   greater than 95%, we considered this egg as a candidate for being from this female. To

325   corroborate this conclusion, we used non-phenotypic data e.g. laying dates, laying locality and

326   host species.

327

328   *Phenotypic-genotypic similarity*

329   We had genetic samples for 9 of the adult females, allowing us to create a genetic distance matrix.

330   To compare the phenotypic-genotypic similarity between these females and their eggs, we

331   created a trait distance matrix by taking means of the phenotypic parameters from their egg data,

332   and then using Euclidean distance as the distance metric. We compared the genetic distance

333   matrix with the trait distance matrix using a Mantel test, a statistical test of the correlation between

334   two matrices, implemented in the vegan package in R using the Kendall method (as this is most

335   appropriate for a small dataset). We also split the phenotype data into different components

336   (spectral, pattern and shape) and calculated the phenotype-genotype similarities for each of these

337   components separately, to test whether different aspects of the egg phenotype are differentially

338   correlated with the female genotypes.

339

340   **Results**

341   *Within- and between-female variability in egg appearance*

342   Within-female variance was variable, with some females having very similar eggs (e.g. female 13

343   - within-female variance = 0.33, Fig. 2 in Supplementary material) and others having relatively

344   high variance (e.g. female 29 - within-female variance = 1.31, Fig. 4 in Supplementary material).

345   The between-female variance (comparing across females, using an "average egg" for each

346   female) was 1.832 (mean of trait standard deviations, n = 11 traits; SD = 1.021). Overall, within-

347   female variance (mean = 0.850, SD = 0.295) was smaller than between-female variance (one

348   sample t test, t = 14.87, df = 19, p < 0.001). Variability in the egg appearance is also visible in Fig.

349   1 where the two most informative variables in the random forest analysis (PC2 for pattern and

350   PC2 for spectral data), are plotted (see below and Table 2).

351
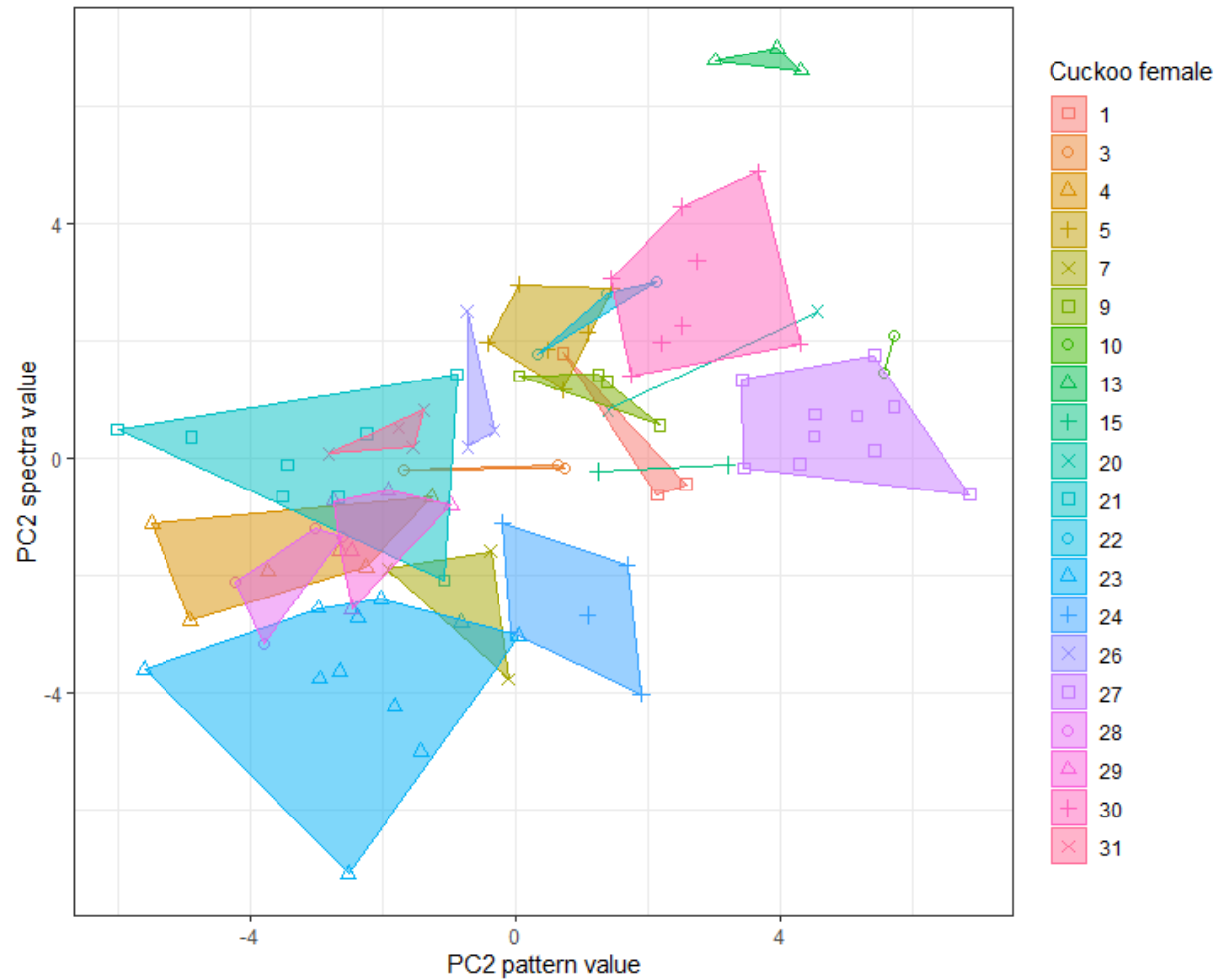
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Figure 1 Values for individual eggs on the two most important PC variables (according to the random forest model), grouped by cuckoo female. PCA2 pattern variable indicates egg skew and PC2 spectra variable indicates blueness/greenness of eggs (for details, see Table 2).

366

367

Human assessment

Participants with some experience of working with biological data performed better at the grouping task than those with no experience, though there is no clear evidence that specific experience of working with cuckoo eggs is beneficial (Table 1).

372   *Table 1. Cluster similarities of egg sorting performed by humans both without knowledge (when*

373   *they did not know the number of females) and with a known number of females.*

Group	No knowledge	Known number of females
No experience (n = 5)	0.225 (0.066)	0.241 (0.041)
Non-specific experience (n = 3)	0.502 (0.170)	0.496 (0.057)
Specific experience (n = 4)	0.417 (0.050)	0.456 (0.158)

374   *Mean cluster similarity (and SD in brackets) is presented for each category.*

375

376   *Unsupervised learning*

377   Clustering using unsupervised hierarchical learning gave a cluster similarity value of 0.452; similar

378   to that of experienced human observers, but better than inexperienced observers.

379

380   *Supervised learning (random forest analysis)*

381   *Female clustering*

382   Clustering using supervised random forest analysis (with a leave-one-out protocol) led to good

383   classification, with a mean of 77.08/95 (81.1%) of eggs correctly assigned to their genetic parent.

384   The cluster similarity had a mean of 0.61 (SD = 0.03), higher than both experienced human

385   assessment and unsupervised learning.

386

387   We assessed variable importance (Table 2) using a full model including all data. PC2 for pattern

388   was the most important variable for classification, and the variables loading onto this PC were

389   predominantly those for the 'skew' of the pattern. PC2 for spectra was also important, with this

390   variable being influenced by the 'blueness/greenness' of the egg. Finally, PC3 and PC1 for shape

391   were also informative. The variables loading onto these PCs were the length, width, volume and

392   surface area of the egg.

393

394 *Table 2 The importance of individual variables in egg clustering using random forest analysis.*

Variable	Mean decrease in accuracy	Main PCA loadings
PC2_pattern	28.42	Skew values at pattern energy scales 1, 0.707, 0.5, 0.3536, 0.25, 0.1768, 0.125, 0.08839, 0.0625, 0.04419
PC2_spectra	26.8	426, 447, 468, 489, 510, 531nm
PC3_shape	23.81	Length, max width
PC1_shape	21.37	Length, max width, volume, surface area
PC1_spectra	19.79	342, 552, 573, 594, 636, 678, 699nm
UV_shape	19.36	-
PC2_shape	16.91	Ellipse deviation
PC1_luminance	15.42	Luminance sections 1, 2 and 3, standard deviation sections 1, 2 and 3
PC3_pattern	15.18	Pattern energy scales 1, 0.7071, 0.5, 0.3536, 0.04419, 0.03125, 0.0221
Brightness	12.9	-
PC1_pattern	11.23	Pattern energy scales 0.3536, 0.25, 0.1768, 0.125, 0.08839, 0.0625, total pattern energy, total pattern energy in segment 2

395 *Variables with larger mean decrease in accuracy are more important for classifying the data*

396 *(mean decrease in accuracy is a measure of how much the accuracy of the random forest*

397 *decreases due to the exclusion/permutation of a single variable). The main PCA loadings are*

398 *those that were greater than +/- 0.25.*

399

400

401   *Same/different analysis*

402   For our labelled data, on average, 77.46/95 (81.5%) of eggs were matched with themselves.

403   During the training phase, the model was always trained with 'same' pairs that consisted of two

404   different eggs from the same female, so this was done as a test of whether the model was able

405   to recognise two truly identical eggs as coming from the same female.

406   In addition, on average 73.50/95 (77.4%) eggs were uniquely matched with other eggs laid by the

407   same female.

408   Out of 87 unlabelled eggs, the model was able to reliably (on 95% of runs) identify 22 as belonging

409   to a labelled female (9 eggs assigned to female 5, 5 eggs to female 27, 3 eggs to female 13, and

410   1 egg to each of females 4, 21, 28, 29, 30).

411

412   *Phenotypic-genotypic similarity*

413   There was no significant relationship between overall female egg phenotype distance and female

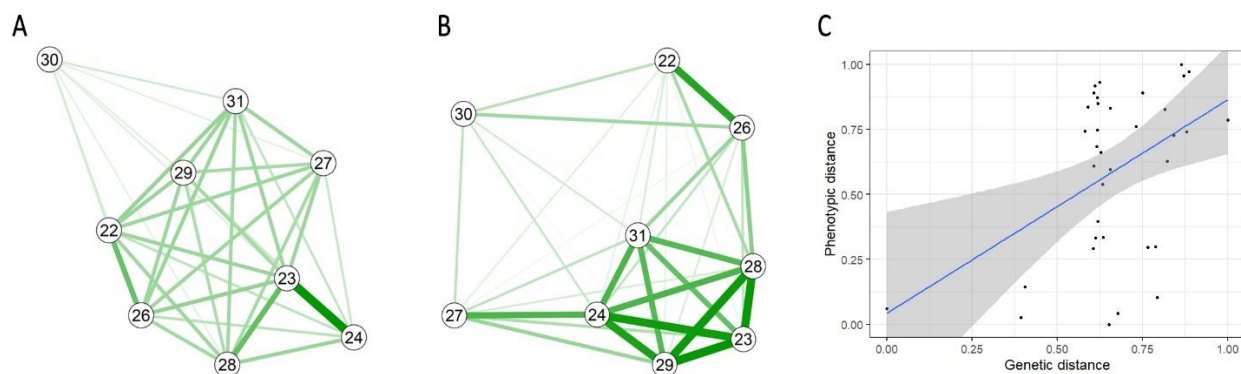414   genetic distance (Mantel test r = 0.1968, p = 0.098, Fig. 2).

415

416

417   *Figure 2 Phenotypic distances of nine average eggs laid by nine genotyped common cuckoo*

418   *females (A) and their genetic distances (B). Thicker green lines denote higher phenotypic and*

419   *genetic similarity. Correlation between phenotypic and genetic distances (C).*

420

421   When considering each aspect of phenotypic similarity separately, both pattern/luminance and

422   shape distance metrics did not correlate with genotypic similarity (r = 0.0254, p = 0.3861 and r =

423   -0.2317, p = 0.9256 respectively, Fig. 3A-D). However, spectral similarity did correlate with genetic
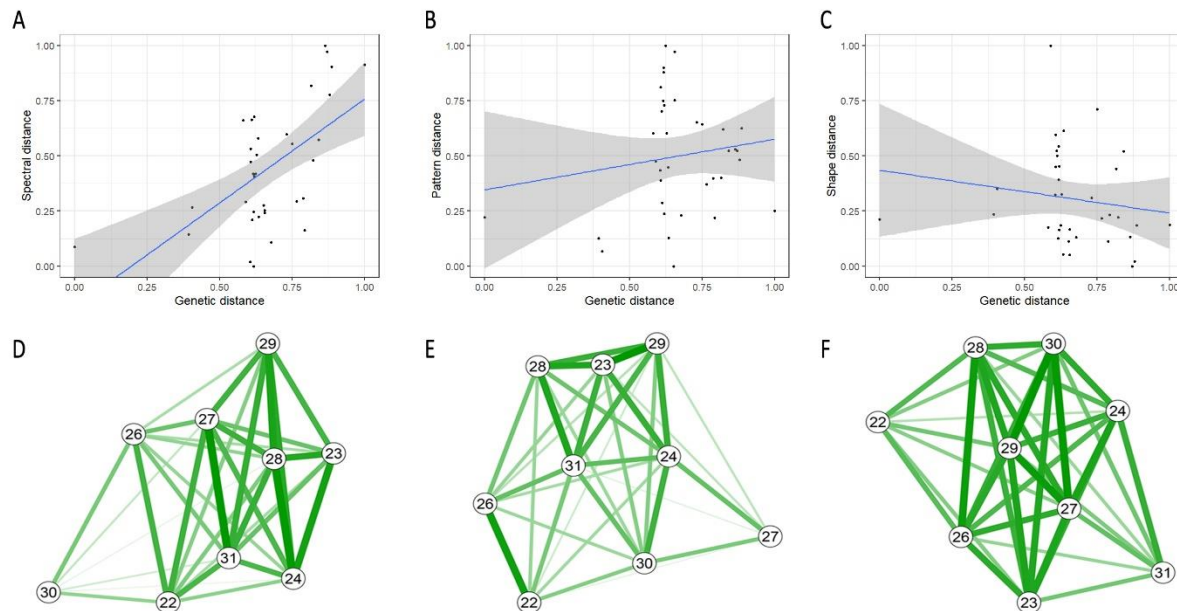
424   similarity (r = 0.356, p = 0.037, Fig. 3E-F).

425

426

427   *Figure 3 Correlation between spectral (A), pattern/lumiance (B) and shape (C) distances,*

428   *respectively and genetic distances. Individual phenotypic distances of average eggs laid by nine*

429   *genotyped common cuckoo females: spectral (D), pattern/luminance (E) and shape (F) distances.*

430

431

Discussion

433   The results of our study support the 'constant egg-type hypothesis' predicting that individual

434   cuckoo females lay eggs with a relatively constant appearance (Latter 1902, Chance 1940, Baker

435   1942, Lack 1968, Wyllie 1981, Moksnes et al. 2008). We confirmed that the within-clutch variation

436   of cuckoo egg appearance is significantly lower than between-clutch variation. This has also been

437   observed in other bird species and several adaptive explanations have been proposed for this

438   phenomenon, such as easier recognition of the parasitic egg by hosts (Øien et al., 1995),

439   recognition of an individual's own clutch in colonially-breeding birds (Hauber et al., 2019) or

440   signalling the female quality (Moreno & Osorno, 2003). Moreover, it has been shown that diet has

441   only a small effect on eggshell colour and that this trait is more affected by maternal identity,

442   suggesting that egg colour may carry information about intrinsic properties of the female

443   (Dearborn, Hanley, Ballantine, Cullum, & Reeder, 2012). Therefore, it seems that there is the

444   potential to use egg appearance to identify individual bird females.

445

446   Here, we demonstrated that the unsupervised computer-vision based classifier can outperform

447   human participants (especially inexperienced ones) in assigning cuckoo eggs to correct female

448   clusters. Human egg classifiers with experience of handling natural eggs (either cuckoo or of other

449   species) were able to more accurately sort them compared to people without this experience

450   (Table 1). This may indicate that experienced observers have some knowledge of which cues are

451   likely to be useful. They may also be more likely to be practiced at this type of fine discrimination

452   task, or be more motivated to take part, given their interest in biology. Interestingly, knowing the

453   number of groups (here: cuckoo females that laid eggs) did not increase sorting accuracy

454   substantially.

455

456   The automatic hierarchical clustering method showed very similar results to experienced human

457   classifiers, while supervised random forest analysis showed considerably better results and 81%

458   of cuckoo eggs were assigned correctly. This suggests that in some cases, automatic assessment

459   methods may be preferable to human assessment. Detailed consideration of the clusters created

460   by humans and the automatic methods showed that the same females were problematic for both

461   clustering methods, probably reflecting phenotypic overlap between some individuals (all sorting

462   results can be found in Supplementary Material). Our results showed that one of the pattern

463   characteristics (skew), blueness of colour and finally egg size were the most important parameters

464   that helped the automatic method to cluster eggs the most accurately. The slight improvement in

465   clustering accuracy for the automatic methods over human assessment may reflect the use of

466   features that humans are not able to see (e.g. the UV peak of the spectra). Therefore, we would

467   recommend automatic assessment over human assessment where possible.

468

469   A previous study suggested that closely related cuckoo females may lay eggs that are

470   indistinguishable from each other (Moksnes et al., 2008). Our limited dataset of nine caught

471   females for which we calculated genetic distances partially supports this idea as we found that

472   the background colour of eggs was more similar between closely related females. However,

473   pattern and dimension distances between females did not correlate with their genetic distances.

474   As an interesting example, we caught two closely related females, mother (female 23) and

475   daughter (female 24), and we photographed and measured the colour of their eggs. From the

476   photographs, we can see that their eggs look very similar in the colour and spotting pattern;

477   however, they differ considerably in size (Fig 3 in Supplementary material). Similarly, we can also

478   see the resemblance in egg colour and pattern (but not in size) between other closely related

479   females (females 23 vs 28 and females 22 vs 26; Fig. 3 and 4 in Supplementary material).

480   Previous studies showed that all investigated egg features - colour, spotting pattern and also size

481   have high heritability (Wei et al., 1992; Collias, 1993; Gosler et al., 2000; Christians, 2002;

482   Morales et al., 2010). Our results indicate that the background colour might be a more heritable

483   trait than spotting pattern and egg size or shape, which supports the idea that egg colour is of

484   vital importance for egg recognition (Avilés et al., 2010; Spottiswoode & Stevens, 2010; Michal

485   Šulc, Procházka, Capek, & Honza, 2016). Since several studies reported that hosts and even

486   parasites themselves also use spotting pattern (López-de-Hierro & Moreno-Rueda, 2010;

487   Spottiswoode & Stevens, 2010; de la Colina, Pompilio, Hauber, Reboreda, & Mahler, 2012) or

488   egg size (Marchetti, 2000; Spottiswoode, 2013) when recognizing and eliminating parasitic eggs,

489   we still expect relatively high heritability of these egg traits in brood parasites. We suspect that

490   the insignificant relationship between genetic distance and phenotypic distance in spotting pattern

491   and size is only a matter of our limited sample size. A Larger sample size, including more mother-

492   daughter pairs, is needed to truly estimate heritability values of individual egg traits (de

493   Villemereuil, Gimenez, & Doligez, 2013). The lack of significant correlation between egg shape

494   and genetic similarity may also be explained by the fact that egg size often reflects the size of

495   laying females (Larsson & Forslund, 1992; Nager & Zandt, 1994), which depends on the genetic

496   contribution of both parents and therefore might differ more even in closely related females.

497   Moreover, even within one host population, cuckoos are raised by host parents that vary in their

498   provisioning care (Požgayová, Piálková, Honza, & Procházka, 2018), which may also influence

499   the body size of cuckoo females in adulthood. Finally, some studies showed there is a positive

500   relationship between food availability and egg size (reviewed in Christians 2002). Consequently,

501   since egg size and shape may even differ in such closely related females, these traits may be

502   used for the separation of their eggs. And indeed, this is what we observed in several of our

503   human participants and also in computer analysis where separation of mother and daughter eggs

504   was relatively straightforward presumably because of the apparent difference in the size and

505   shape (see Supplementary material).

506

507   Although our results are in concordance with a previous study showing that the visual appearance

508   of cuckoo eggs cannot be used to assign them reliably to individual females without genetic data

509   (Moksnes et al. 2008), here we present a new approach that uses both genetic and phenotypic

510   information. We used this method for assigning cuckoo eggs for which we did not have genetic

511   data (because they were ejected by hosts or predated), allowing us to assign 22 eggs (out of 87)

512   to eight known females. This method seemed to work well for females that laid very distinctive

513   eggs and therefore results will strongly depend on within- and between-clutch variation. We may

514   expect better results of the method in species where between-clutch variation substantially

515   exceeds the within-clutch variation. It must also be noted that the accuracy of the assignment will

516   increase with the relative number of (genetically and phenotypically) analysed samples in the

517   study area that are used for the training dataset, because broad sampling will reduce the chance

518   that an unsampled egg that has been laid by a completely new female will be assigned to an

519   existing (incorrect) female. Finally, it is important to apply other information (laying date and laying

520   area) to eliminate potential incorrect assignments. However, in our dataset, we did not find any

521   such discrepancies for the 22 eggs that were automatically assigned based on their phenotypes.

522

523   We conclude that egg appearance alone cannot be used to identify individual cuckoo females.

524   Clusters created either by people using egg photographs or by the computer using spectral and

525   image data did not fully correspond to the true female identity acquired from molecular analyses,

526   though the supervised automatic assessment was the most accurate for classification. Our results

527   support the idea that more closely related females lay eggs more similar in their colour. However,

528   the size and shape of the eggs seems to be the least heritable trait, which may substantially help

529   to distinguish even between eggs of closely related cuckoo females. We advocate genetic

530   analysis to be used for determination of maternity in this species. However, in sufficiently sampled

531   systems, supervised analytical methods that use egg visual features might additionally help to

532   broaden sample sizes, which may be very desirable for studying biological questions (see e.g.

533   Koleček et al. 2020 in prep). We encourage researchers investigating inter- and intra-specific

534   brood parasitism to use this low-cost and ethically more appropriate method of individual

535   identification. Since a similar technique has been successfully used in non-parasitic species

536   (Höltje et al., 2016), identification of laying females using egg appearance therefore has the

537   potential to be of widespread use.

538

539

540

Ethical note

This study was carried out with the permission of the regional nature conservation authorities (permit numbers JMK: 115874/2013 and 38506/2016; MUHOCJ: 41433/2012/OŽP, 34437/2014/OŽP, and 14306/2016/OŽP). The fieldwork adhered to the animal care protocol (experimental project numbers 039/2011 AV ČR and 3030/ENV/17-169/630/17) and to the Czech Law on the Protection of Animals against Mistreatment (license numbers CZ 01272 and CZ 01284). This study was carried out with the permission of the regional nature conservation authorities (permit numbers JMK: 115874/2013 and 38506/2016; MUHOCJ: 41433/2012/OŽP, 34437/2014/OŽP, and 14306/2016/OŽP).

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