Genetic differentiation among sympatric cuckoo host races: males matter

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Generalist parasites regularly evolve host-specific races that each specialize on one particular host species. Many host-specific races originate from geographically structured populations where local adaptations to different host species drive the differentiation of distinct races. However, in sympatric populations where several host races coexist, gene flow could potentially disrupt such host-specific adaptations. Here, we analyse genetic differentiation among three sympatrically breeding host races of the brood-parasitic common cuckoo, Cuculus canorus. In this species, host-specific adaptations are assumed to be controlled by females only, possibly via the female-specific W-chromosome, thereby avoiding that gene flow via males disrupts local adaptations. Although males were more likely to have offspring in two different host species (43% versus 7%), they did not have significantly more descendants being raised outside their putative foster species than females (9% versus 2%). We found significant genetic differentiation for both biparentally inherited microsatellite DNA markers and maternally inherited mitochondrial DNA markers. To our knowledge, this is the first study that finds significant genetic differentiation in biparentally inherited markers among cuckoo host-specific races. Our results imply that males also may contribute to the evolution and maintenance of the different races, and hence that the genes responsible for egg phenotype may be found on autosomal chromosomes rather than the female-specific W-chromosome as previously assumed.

Keywords: brood-parasitism; genetic differentiation; host-specific races

1. INTRODUCTION

Coevolution, the reciprocal evolutionary change in interacting species, is continually reshaping traits within and between species, and is one of the central biological processes organizing the web of life [1,2]. Coevolutionary interactions between parasites and their hosts can drive rapid genetic changes in both species, and may ultimately result in speciation [3,4]. Generalist parasites that use multiple host species regularly evolve host-specific races, which originate from geographically structured populations where local adaptations to different host species drive the differentiation of distinct parasite races [4]. However, when different host-specific races coexist within a restricted geographical area, gene flow could potentially disrupt the host-specific adaptations. This process, called gene swamping, typically results in the loss of polymorphism and fixation of alleles showing the best average reproductive success across all populations [5]. A particularly interesting species in this respect is the generalist avian brood-parasitic common cuckoo Cuculus canorus, which uses a variety of different passerine species across a wide range of shifting habitats, and has evolved several host-specific races (gentes) mimicking the appearance of egg from their respective host species [6–10].

The common cuckoo is an obligate brood-parasite that never builds a nest of its own, but lays its eggs in the nest of other bird species. Soon after hatching, the young cuckoo evicts the hosts own eggs and nestlings, thereby becoming the sole beneficiary of the foster parents care. The host parents thus not only suffer the loss of their own offspring, but also waste time and energy in raising an unrelated foster offspring. This enormous fitness cost generates a strong selection on the host parents for recognizing and ejecting the cuckoo egg. In turn, this generates a strong selection for egg mimicry in the parasitic cuckoo to avoid rejection, resulting in an ever escalating coevolutionary arms race between the host and parasite, eventually giving rise to highly specialized host races mimicking their specific host species [11]. Across Europe, there are at least 17 distinct cuckoo egg morphs mimicking the egg appearance of one specific species or a small range of similar host species [9,12–14]. Individual cuckoo females lay eggs that are highly repeatable in coloration and patterning [15], and appear to be fairly consistent in choice of host species [16,17]. On the other hand, males are known to mate with females from different host races and sire offspring in more than one host species [16,18].
The maintenance of host-specific races in the cuckoo is an evolutionary puzzle, which was recognized 100 years ago [19] and has continued to intrigue researchers ever since [16, 20–25]. With the discovery of females being the heterogametic sex in birds, a simple theoretical solution emerged. If the genes for eggshell appearance are located on the female-specific W-chromosome, fathers would have no influence on the egg appearance of their daughters. Therefore, host-specific mimicry would be maintained simply by female cuckoo nestlings imprinting on their foster species and preferring this species themselves when reaching maturity [20, 23]. Each daughter would inherit her egg phenotype directly from her mother and host rejection of dissimilar eggs would quickly select for cuckoo female lines (matrilines) having mimetic eggs, even in sympatric populations harbouring several host-specific races. Consistent with this suggestion, eggshell spotting pattern of individual females was found to resemble those of both their mothers and maternal grandmothers, but not their paternal grandmothers in great tits Parus major [26]. However, another study found no support for matrilineal inheritance of spotting pattern in the brood-parasitic shiny cowbird Molothrus bonariensis [27]. In the domestic chicken Gallus gallus and Japanese quail Coturnix japonica, F₁ intercrosses and backcrosses clearly show that eggshell background colour is controlled by at least two independent autosomal loci [28, 29]. Moreover, analyses of natural crosses in the egg polymorphic passerine village weaver Ploceus cucullatus were also consistent only with a model of two independent autosomal loci controlling the inheritance of background colour [30]. In general, the small and uniform avian W-chromosome contains few functional loci [31, 32], and it is unlikely that it carries genes coding for all aspects of egg phenotype.

The expectations of theoretical models explaining the evolution and maintenance of host-specific races and egg mimicry are highly dependent on the way egg appearance is inherited. Whereas matrilineal inheritance only requires that female cuckoo nestlings imprint on their host species and prefer this species themselves when starting breeding to evolve egg mimicry, biparental inheritance also requires that the females mate assortatively with males from their own host race. Gibbs et al. [24] found significant genetic differentiation for maternally inherited mitochondrial DNA markers, but not for biparentally inherited microsatellite markers among cuckoo host races in Great Britain and Japan, suggesting that females are host-specific but mate indiscriminately with males from different host races. Moreover, the lack of genetic differentiation for microsatellite markers among host races indicates that males are not contributing to the maintenance of the specific races. However, in Great Britain, sampling was carried out over a large area not conducive for detecting local patterns of gene flow among sympatric host races, and one of the two host races in Japan only very recently became a cuckoo host and hence may not have had time to differentiate significantly [24]. Here, we investigate genetic differentiation among three cuckoo host-specific races breeding sympatrically at a very small spatial scale in northwestern Bulgaria. We predicted that if males contribute to the egg colour of their daughters, assortative mating should be adaptive and result in genetic differentiation of both biparentally inherited microsatellite DNA markers as well as maternally inherited mitochondrial DNA markers in cuckoo nestlings found in the three host species.

2. MATERIAL AND METHODS

(a) Study area and field procedures

Data collection was carried out in an area of ca 10 km² in the vicinity of the village Zlatia (43°46’N, 23°30’E), northwestern Bulgaria. We systematically searched for cuckoo host nests from early May until late July during 2005–2009. Within the study area, three common passerine species are regularly parasitized. The marsh warbler Acrocephalus palustris breeds in various types of herbaceous vegetation, the great reed warbler Acrocephalus arundinaceus in dense reed beds and the corn bunting Miliaria calandra in a mixture of open grassland interspersed with trees and bushes [33, 34]. The different habitats occupied by the different hosts are distributed in a patchy mosaic, and do not form three distinct geographically separated areas. Parasitism rates vary from 9 per cent in the corn bunting [33], 28 per cent in the marsh warbler [34] to 40 per cent in the great reed warbler (own 2010, unpublished data). The host species differ in body size with corn buntings being 40 g, the great reed warblers 33 g and marsh warblers 11 g [35]. We collected blood samples (5–25 μl) from cuckoo nestlings found in the nest of each host species by puncturing the brachial or femoral vein. The blood samples were preserved in 96 per cent ethanol for subsequent genetic analyses.

(b) Genetic analyses

DNA was extracted from the blood samples using EZNA blood DNA kit (Omega Bio-Tek Inc, Norcross, USA). The genetic markers were amplified by polymerase chain reaction on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA) and run on a 3130XL Genetic Analyser (Applied Biosystems, Foster City, USA). For the mitochondrial DNA analysis, we sequenced the same 411 basepair (bp) portion of the left-hand hypervariable control region (CCRL1A; 5'-CTGATAATACATGATGAGCT CGT-G' and CCRH1; 5'-CTGAAATAGTATGAATGTATCT GTG-3') as used by Gibbs et al. [24]. For the autosomal DNA analyses, we first genotyped the same 10 markers used by Gibbs et al. [24]. However, one marker (Ccy3) turned out to deviate from Hardy–Weinberg equilibrium in our sample, and therefore, had to be excluded. In order to increase the power of assigning half- and full-siblings, we genotyped four more markers, adding up to 13 microsatellites in total (electronic supplementary material, table S1). The mitochondrial sequence data were assembled using the software Geneious v. 4.7.6 [36], and the microsatellite markers were scored using the software Genemapper v. 3.7 (Applied Biosystems, Foster City, USA). To ensure consistency, all genotypes were scored by one person (F.F.).

As we have collected data within a relatively small study area across several years, we may have sampled multiple offspring from the same female, creating a possible problem of pseudoreplication. Therefore, we used the software Colony [37] to determine half- and full-sibling relationships among the sampled cuckoo nestlings. In contrast to most other similar software that only considers pairwise comparisons,
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Colony uses a full-pedigree likelihood approach, which considers the likelihood of the entire pedigree structure and allows for the simultaneous inference of parentage and sibship. Moreover, Colony allows the user to add information on known relationships among the offspring to increase the probability of correctly assigning sibship. We added information on mitochondrial DNA by defining that two offspring having different mitochondrial haplotypes could not possibly be maternal half-siblings. Moreover, offspring having identical mitochondrial haplotypes but that hatched from two very different looking eggs were also designated as being from two different females. The appearance of individual eggs from each cuckoo female is highly repeatable and can be used to assign individual eggs to different females, although different females may produce similar eggs [15].

In order to test for departure from Hardy–Weinberg equilibrium and linkage disequilibrium among the microsatellite markers, we randomly selected one offspring per cluster to avoid pseudoreplication. We used exact tests as implemented in the software Arlequin v. 3.11 [38] to test for departure of Hardy–Weinberg equilibrium for each marker across populations. One marker, Ccm2, showed a significant departure from Hardy–Weinberg equilibrium and was, therefore, excluded from further analyses (electronic supplementary material table S1). We used the software Fsstat v. 2.9.3.2 [39] and the genotypic disequilibrium function to test for linkage disequilibrium. We found no evidence of genotypic disequilibrium between any pair of our microsatellite markers (data not shown).

We used Arlequin also for calculating overall and pairwise $F_{ST}$ values between the host races for the mitochondrial marker (gamma-corrected (0.08) Kimura 2, 10,000 permutations). As maternal siblings share the same haplotype, we included only one offspring per female to avoid pseudoreplication. However, for autosomal microsatellite DNA, offspring from the same female are not equal in allelic representation. Therefore, we used a hierarchical $F_{ST}$ analysis as implemented in the package Hierfstat [40] of the statistical software R [41] to control for pseudoreplication. This approach allows for the inclusion of all offspring while controlling statistically for female/male identity (J. Goudet 2010, personal communication). In a hierarchical $F_{ST}$ analysis, each level of population structure is tested independently of the effect of the lower levels in the hierarchy. Hierfstat uses a likelihood ratio G-statistic and randomizes the diploid genotypes rather than the alleles [40], which is most appropriate for diploid organisms [42].

We used the software Network v. 4.5.1.6 (www.fluxus-engineering.com) to visualize the mitochondrial haplotype genealogies.

3. RESULTS

(a) Sibship reconstruction

We successfully genotyped 79 cuckoo nestlings (mean number of microsatellite markers = 12.9, range 12–13) originating from 25 great reed warbler nests, 23 marsh warbler nests, 30 corn bunting nests and one reed warbler nest. After running Colony, we removed 10 nestlings that showed a probability below 0.95 (range: 0.126–0.933) of being either full- or half-sibling with one or a group of offspring. This ensures that our results are conservative since we cannot be entirely sure that all of these nestlings are unrelated to their respective groups, and therefore, poses a problem of pseudoreplication. However, we also ran the analyses including the 10 nestlings as independent family groups. The results remained unchanged and we, therefore, present only the most conservative analyses. All remaining pairs of full- and half-siblings showed a probability above 0.99 of their relationship, and the grouping was consistent in subsequent repeated runs. The 69 cuckoo offspring remaining in the analysis were produced by 30 females and 19 males, of which 15 females and 14 males had more than one offspring in our sample. Females produced on average 2.3 offspring (s.d. = 1.9, range 1–7), whereas males sired on average 3.6 offspring (s.d. = 2.6, range 1–11) within our dataset (Mann–Whitney test: $W = 189.5$, $p = 0.043$).

Of the adults producing more than one offspring, four females (27%) and nine males (64%) were polygamous and had offspring with more than one partner (Fisher exact test, $p = 0.002$). Furthermore, one female (7%) and six males (43%) had offspring in nests of more than one host species (Fisher exact test, $p = 0.035$; table 1) and were, therefore, not strictly host-specific. None of the males or the female had offspring in more than two host species (table 1). However, the majority of offspring were produced within one host race and only one of 54 (2%) maternal descendants, and six of 64 (9%) paternal descendants were fostered outside their putative parents’ host species (Fisher exact test, $p = 0.12$).

(b) Mitochondrial genetic structure

There was significant genetic differentiation among the female host races in the 411 bp mitochondrial control region ($F_{ST} = 0.17$, $p = 0.035$). A pairwise $F_{ST}$ comparison between the three host races revealed that the marsh warbler cuckoos were significantly different from the two other races, whereas the corn bunting and great reed warbler cuckoos were not significantly different from each other for this marker (table 2a).

The haplotype network disclosed a rather intricate pattern with no clear grouping of any host race (figure 1). Haplotype sharing among host races, i.e. the same haplotype being found in more than one host race, occurred in three (20%) of 15 haplotypes found in our sample.

(c) Microsatellite genetic structure

The hierarchical $F_{ST}$ analysis showed significant genetic differentiation among the female host races also for the microsatellite markers ($F_{ST} = 0.025$, $p < 0.001$, $n = 69$ offspring, 30 females). Moreover, pairwise hierarchical
Table 2. Pairwise $F_{ST}$ values and the corresponding levels of significance (in parentheses) between the three host races for (a) the 411 basepair mitochondrial control region, $n = 9$ corn bunting, nine great reed warbler and 12 marsh warbler females; (b) microsatellite markers controlling for female identity, $n = 25$ nestlings from nine corn bunting females, 20 nestlings from nine great reed warbler females and 24 nestlings from 12 marsh warbler females; (c) microsatellite markers controlling for male identity, $n = 26$ offspring from eight corn bunting males, 20 nestlings from five great reed warbler males and 23 nestlings from six marsh warbler males; and (d) microsatellite markers controlling for cluster identity, $n = 25$ offspring from four corn bunting clusters, 20 nestlings from five great reed warbler clusters and 24 nestlings from six marsh warbler clusters. (Statistically significant values are emphasized in bold. To control for multiple comparisons of the microsatellite data, we included all nine $p$-values in a sequential Bonferroni correction and all tests remained significant.)

<table>
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<tr>
<th></th>
<th>corn bunting</th>
<th>great reed warbler</th>
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<td>(a) mtDNA</td>
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<tr>
<td>great reed warbler</td>
<td>0.05 (0.50)</td>
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<td>marsh warbler</td>
<td>0.24 (0.028)</td>
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<td>(b) female</td>
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<td>great reed warbler</td>
<td>0.030 (&lt;0.001)</td>
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<td>marsh warbler</td>
<td>0.013 (0.009)</td>
<td>0.035 (0.007)</td>
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<tr>
<td>(c) male</td>
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<tr>
<td>great reed warbler</td>
<td>0.032 (&lt;0.001)</td>
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<td>marsh warbler</td>
<td>0.009 (0.018)</td>
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<td>(d) cluster</td>
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<tr>
<td>great reed warbler</td>
<td>0.049 (0.001)</td>
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<tr>
<td>marsh warbler</td>
<td>0.009 (0.011)</td>
<td>0.043 (0.012)</td>
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$F_{ST}$ analyses revealed that all three female host races were significantly differentiated from each other (table 2b). We also ran a hierarchical $F_{ST}$ analysis controlling for male identity and host race, and found a significant genetic differentiation ($F_{ST} = 0.025$, $p < 0.001$, $n = 69$ offspring, 19 males). Pairwise hierarchical analyses showed that all three male host races were significantly differentiated from each other (table 2c). Finally, we controlled for both female and male identity simultaneously by randomizing offspring within clusters (offspring in different clusters do not share any parent) and the genetic differentiation remained significant ($F_{ST} = 0.032$, $p < 0.001$, $n = 69$ offspring, 15 clusters). Pairwise hierarchical analyses corroborated that all three host races were significantly differentiated from each other (table 2d). Finally, we selected one offspring per female and ran the analyses in ARLEQUIN using the same settings as for the mitochondrial marker. Also here we found a significant genetic differentiation ($F_{ST} = 0.018$, $p = 0.027$, $n = 30$ females).

4. DISCUSSION

We found significant genetic differentiation in both biparentally inherited microsatellite DNA and maternally inherited mitochondrial DNA in a sympatric population of cuckoo host races. To our knowledge, this is the first study that finds significant genetic differentiation in biparentally inherited microsatellite markers among cuckoo host races. Our results imply that assortative mating occurs regularly and that males thus are likely to contribute to the evolution and maintenance of host-specific races in the cuckoo. Moreover, this raises the possibility that egg mimicry can evolve in the presence of paternal effects on egg phenotype and hence that the genes responsible for egg phenotype may reside on autosomal chromosomes, rather than the female-specific W-chromosome as commonly assumed.

Our finding of a significant autosomal genetic differentiation among host races is further strengthened by the degree of sympathy and small size of our study area. The entire study area is less than 10 km$^2$, which should provide all cuckoo females with ample opportunity to mate with any male and lay their eggs in any one of the three host species. However, only four females (27%) produced offspring with more than one male and only one female (7%) laid their eggs in more than one host species. On the other hand, males tended to be more promiscuous and less host-specific than the females with nine males (64%) being polygamous and six males (43%) siring offspring in nests of more than one host species. These results are very similar to what Marchetti et al. [16] found in Japan, where 8 per cent of females and 37 per cent of males had offspring in nests of more than one host species. However, if we look at the number of offspring found in different host species, only 2 per cent of female and 9 per cent of male descendants were fostered outside their parents’ putative host species, suggesting that each individual produces most of its offspring in one host species, and only one or a few offspring in another host species. Hence, male gene flow among cuckoo host races is actually less than suggested by the number of males siring offspring in two different host species.

Surprisingly, only two of the three female host races showed significant genetic differentiation for the mitochondrial control region, whereas all three of them were genetically differentiated for the microsatellite markers. The mitochondrial haplotype network disclosed a relatively intricate pattern, where no host race appears to be clearly preceding the other two (figure 1). There were also several cases of haplotype sharing (20%) among the host races, and together this indicates multiple origins of each cuckoo host race, which corroborates the conclusion of Gibbs et al. [24]. The corn bunting appears to be the most recent host in our study area and it shows the lowest level of egg rejection and is parasitized by cuckoos showing the lowest level of egg mimicry [43]. The corn bunting is, therefore, most likely to accept eggs from other host races, and it is possible that a few successful female host switches from great reed warblers to corn buntings have introduced a common pool of mitochondrial haplotypes, but still kept the frequency of common microsatellite alleles different for the two host races.

How cuckoos select their mates is currently unknown but several plausible mechanisms for assortative mating can be depicted. First, three mechanisms have been proposed to explain how cuckoos locate their preferred host that may each result in some level of assortative mating. Cuckoos may select their host based on: (i) the density of the host species that reared them—the host preference hypothesis; (ii) the habitat in which they were reared—the habitat imprinting hypothesis; or (iii) through natal philopatry (e.g. [13]). Second, several specific mechanisms may also result in assortative mating. For example, time of breeding is somewhat different among the host species,
which could result in ‘isolation by time’ between the cuckoo parasitizing them [44]. Among our 32 females, the corn bunting cuckoos laid on average on 8 May, great reed warbler cuckoos on 25 May and marsh warbler cuckoos on 26 May. Hence, the corn bunting cuckoo could partly be isolated by time of breeding from the two other races. Furthermore, a recent study discovered that cuckoo males from distant populations from the same habitat type had more similar vocal calls than those of nearby populations from different habitats [45]. Hence, if females prefer the call of male cuckoos from their own habitat, non-random mating may result. Also, growth rate and fledging mass of cuckoo nestlings vary among the different host species [46]. If this size difference is also reflected in adults, there is a possibility of assortative mating based on body size. Among the three host species studied here, marsh warblers are only half the size of the two other species, which are more similar in size [35]. Unfortunately, we have no information regarding size differentiation between the corresponding cuckoo host races. However, their eggs vary in size, demonstrating size mimicry with their specific host species [43]. Since larger birds generally lay larger eggs [47], it is reasonable to suppose that cuckoos parasitizing the small marsh warbler are smaller than those parasitizing the much larger great reed warbler and corn bunting.

The occurrence of non-random mating also makes it possible that cuckoo host races have innate host-specific adaptations. Innate host-specific adaptations have previously been found in cuckoo nestlings. Davies et al. [48] discovered that cuckoo nestlings found in reed warbler nests were predisposed to respond to the alarm calls of their specific host and that this was not caused by learning. Hence, most probably both males and females show host-specific genetic predispositions at the chick stage, which indicates that other genetic predispositions for host specificity may also exist.

Whereas we found evidence of genetic differentiation among host races both in mitochondrial and nuclear DNA, Gibbs et al. [24] found only significant genetic differentiation in mitochondrial DNA. This may be explained by a number of factors. For example, different markers and different methods were used. One of the 10 initial microsatellite markers used by Gibbs et al. [24] turned out to deviate from Hardy–Weinberg equilibrium in our sample, and therefore, had to be excluded. We also added four new markers to be able to confidently assign sibship correctly. In Japan, Gibbs et al. [24] had the advantage of having DNA from adult females for which radiotracking had disclosed their host preference. However, one of the two host races in Japan exploits a new, recently adopted cuckoo host, and hence may not have had time to diverge at the neutral microsatellite loci. In Great Britain, Gibbs et al. [24] selected only unrelated nestlings, whereas we have used a hierarchical approach to control for female identity. However, a significant differentiation in mitochondrial DNA without concomitant significant differentiation in microsatellite DNA is not necessarily evidence for maternal inheritance of egg phenotype. First, mitochondrial DNA coalesces relatively rapidly, whereas nuclear DNA is a lagging indicator of genetic differentiation (e.g. [49]). Hence, even completely isolated populations may in the beginning show only significant differentiation for mitochondrial DNA. Second, the specific genes affecting egg phenotype may be highly differentiated, whereas most nuclear loci may show little or no differentiation. Hence, even large phenotypic differentiation at specific traits may not be traceable in neutral microsatellite markers. Therefore, failing to find genetic differentiation using a relatively small number of microsatellite markers is not sufficient to conclude that there is no differentiation in nuclear DNA.

If cuckoo egg appearance is in fact biparentally inherited, the theoretical models on evolution of egg mimicry and maintenance of host races will need to be re-evaluated. At least for the background eggshell colour, available data support biparental rather than matrilineal inheritance [28–30]. Moreover, it is unlikely that the small and uniform avian W-chromosome, which in general contains few functional loci [31,32], carries genes coding for all aspects of the egg phenotype. Even though we found evidence for assortative mating among the cuckoo host races in this study, we also observed several cases of interbreeding between the three races, which could possibly create a problem of gene swamping and breakdown of...
the host-specific mimicry [5]. In a recent study, we found that several aspects of the egg phenotype, including both colour and size differ significantly among our three host races [43]. However, this phenotypic variation does not generate three distinctly different morphs, because the three host races overlap in colour space. Indeed, eggs of corn bunting cuckoos, with the lowest degree of egg mimicry, resemble eggs of the two other cuckoo host races more than those of its own host species [43]. Therefore, the current level of host-specific egg mimicry is consistent with some gene flow occurring among these host races. 

Southern [22] recognized that good mimicry occurs only in large tracts of homogeneous habitat, where one host species is predominant, like for example the Hungarian reed-beds with its large population of great reed warblers. Moreover, he noted that in Khasia Hills, India, intermediate egg types were found exactly in the junctions of different habitats. As our hosts in Zlatia, Georgi Dimov and Peter Genadieva Kirkova and Anton Kirkov for their incredible hospitality as our hosts in Zlatia, Georgi Dimov and Peter Genadieva Kirkova and Anton Kirkov for their incredible hospitality as our hosts in Zlatia, Georgi Dimov and Peter Genadieva Kirkova and Anton Kirkov for their incredible hospitality as our hosts in Zlatia.

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Collection of blood samples complied with the legal provisions of Bulgaria.

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