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Genetic architecture facilitates then constrains adaptation in a host–parasite coevolutionary arms race

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In coevolutionary arms races, interacting species impose selection on each other, generating reciprocal adaptations and counter adaptations. This process is typically enhanced by genetic recombination and heterozygosity, but these sources of evolutionary novelty may be secondarily lost when uniparental inheritance evolves to ensure the integrity of sex-linked adaptations. We demonstrate that host-specific egg mimicry in the African cuckoo finch *Anomalospiza imberbis* is maternally inherited, confirming the validity of an almost century-old hypothesis. We further show that maternal inheritance not only underpins the mimicry of different host species but also additional mimetic diversification that approximates the range of polymorphic egg "signatures" that have evolved within host species as an escalated defense against parasitism. Thus, maternal inheritance has enabled the evolution and maintenance of nested levels of mimetic specialization in a single parasitic species. However, maternal inheritance and the lack of sexual recombination likely disadvantage cuckoo finches by stifling further adaptation in the ongoing arms races with their individual hosts, which we show have retained biparental inheritance of egg phenotypes. The inability to generate novel genetic combinations likely prevents cuckoo finches from mimicking certain host phenotypes that are currently favored by selection (e.g., the olive-green colored eggs laid by some tawny-flanked prinia, *Prinia subflava*, females). This illustrates an important cost of coding coevolved adaptations on the nonrecombining sex chromosome, which may impede further coevolutionary change by effectively reversing the advantages of sexual reproduction in antagonistic coevolution proposed by the Red Queen hypothesis.

coevolution | gentes | maternal inheritance | mimicry | W chromosome

Significance

Validating an almost century-old hypothesis, we show that a critical host-specific adaptation in a brood-parasitic bird, mimicry of host egg coloration, is maternally inherited, allowing mothers to transmit specialized mimicry to their daughters irrespective of the father's host species. This genetic architecture, however, is a double-edged sword for parasites: the loss of recombination and heterozygosity as sources of evolutionary novelty likely constrains the parasite from mimicking the full range of color polymorphisms that hosts have evolved as an escalated defense against parasitism. This important tradeoff of asexual inheritance may have relevance for understanding coevolution in other host–parasite systems.

Genetic recombination and heterozygosity generate evolutionary novelty (1, 2) and, therefore, promote adaptation in rapidly changing selective environments, an advantage considered pivotal to the evolution of sex [the Red Queen Hypothesis (3, 4)]. However, this benefit can be lost when genes advantageous to the heterogametic sex arise on or relocate to the minor sex chromosome (e.g., W in birds or Y in mammals), the single copy of which is transmitted largely without recombination. This uniparental and effectively asexual inheritance can protect coadapted genes from disruptive recombination, thereby ensuring faithful transmission of adaptation across generations (5–7). However, eliminating recombination can limit the adaptive potential of Y and W chromosomes and weaken the efficacy of selection (5). This trade-off between the benefits and costs of uniparental inheritance may be especially acute when sex-linked loci control traits under reciprocal selection in dynamic arms races between antagonists. In this study, we demonstrate maternally inherited host-specific adaptation in a brood-parasitic bird and ask how a lack of sexual recombination shapes the coevolutionary arms race with its hosts.

Brood parasites reproduce by laying eggs in the nests of other species, thereby exploiting the parental care behavior of their hosts. Many hosts defend themselves by rejecting foreign eggs from their nests, which has led to the evolution of egg mimicry by parasites (8, 9). In some parasitic species that exploit multiple host species, phenotypically distinct host races (known as gentes in the context of avian brood parasitism) specialize on different host species by mimicking their respective egg phenotypes. The evolutionary puzzle of how such diversity in host-specific adaptations could evolve and be maintained within a single parasitic species was first recognized in 1910 (10). There are two potential solutions to this puzzle. First, females might mate only with males raised by the same host species, generating reproductive isolation and cryptic speciation between host-specialists (11). Alternatively, since female birds are heterogametic, maternal inheritance of egg mimicry genes via the W chromosome could ensure faithful transmission of adaptations from mother to daughter, even if females mate with males raised by different host species. When combined with environmentally determined

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host choice via imprinting (12), maternal genetic inheritance of egg mimicry would thus ensure that the egg phenotype of females is appropriate for the host species they parasitize.

Several previous tests of this elegantly simple maternal inheritance hypothesis, first proposed by Reginald Punnett in 1933 (13, 14), have used mitochondrial DNA (mtDNA) to test whether parasitic females associated with different host species belong to distinct matrilines. Among these studies, two yielded intriguing but mixed results: female common cuckoos, *Cuculus canorus*, laying immaculate blue eggs belong to one matriline, but females in another matriline parasitize and mimic a range of host species with varying egg colors and patterns (15). In greater honeyguides, *Indicator indicator*, highly divergent matrilines are associated with ground- and tree-nesting hosts, respectively, but within each lineage, additional host-specific diversification in egg size and shape is unrelated to more recent matrilineal divergence (16). Thus, whether Punnett's hypothesis can account for the evolution and maintenance of mimicry down to the level of individual host species within a single interbreeding population of parasites remains uncertain.

To test this, we examined an Afrotropical host–parasite system (Fig. 1) in which both hosts and parasites have evolved exquisite diversity in egg phenotypes as the result of an ongoing coevolutionary arms race. The cuckoo finch, *Anomalospiza imberbis*, parasitizes grass-warblers in the genera *Cisticola* and *Prinia*, four species of which occur together at our study site in Zambia (*SI Appendix*, Fig. S1). These hosts are proficient rejecters of eggs that differ from their own in color and pattern, and all have evolved varying degrees of polymorphism in egg phenotypes, resulting in individual egg "signatures" that enhance detection of parasitic eggs (17, 18). For example, tawny-flanked prinias (*Prinia subflava*) lay eggs with blue, white, red, or olive-green background color overlaid with a variety of patterns (9). Correspondingly, cuckoo finches have evolved egg mimicry not only to match the distinct phenotypes of different host species but also to approximate the range of interindividual variation in color and pattern variants within each host species (Fig. 1 and *SI Appendix*, Fig. S2) (17).

We report here analyses of genetic data testing whether maternal inheritance accounts for both levels of adaptation in cuckoo finches: mimicry of multiple host species (i.e., gentes), and mimicry of polymorphic egg phenotypes within host species (i.e., gentes within gentes). We also analyze mtDNA sequence data for the cuckoo finch's most common and most phenotypically diverse host species at our study site, the tawny-flanked prinia, to test our expectation that hosts should retain autosomal inheritance of egg phenotypes because it facilitates the evolution of new egg signatures (phenotypes) in their coevolutionary race with the cuckoo finch. We show that maternal inheritance maintains mimicry of different host species and that, nested within host-specific female lineages, it both facilitates and constrains polymorphic mimicry of

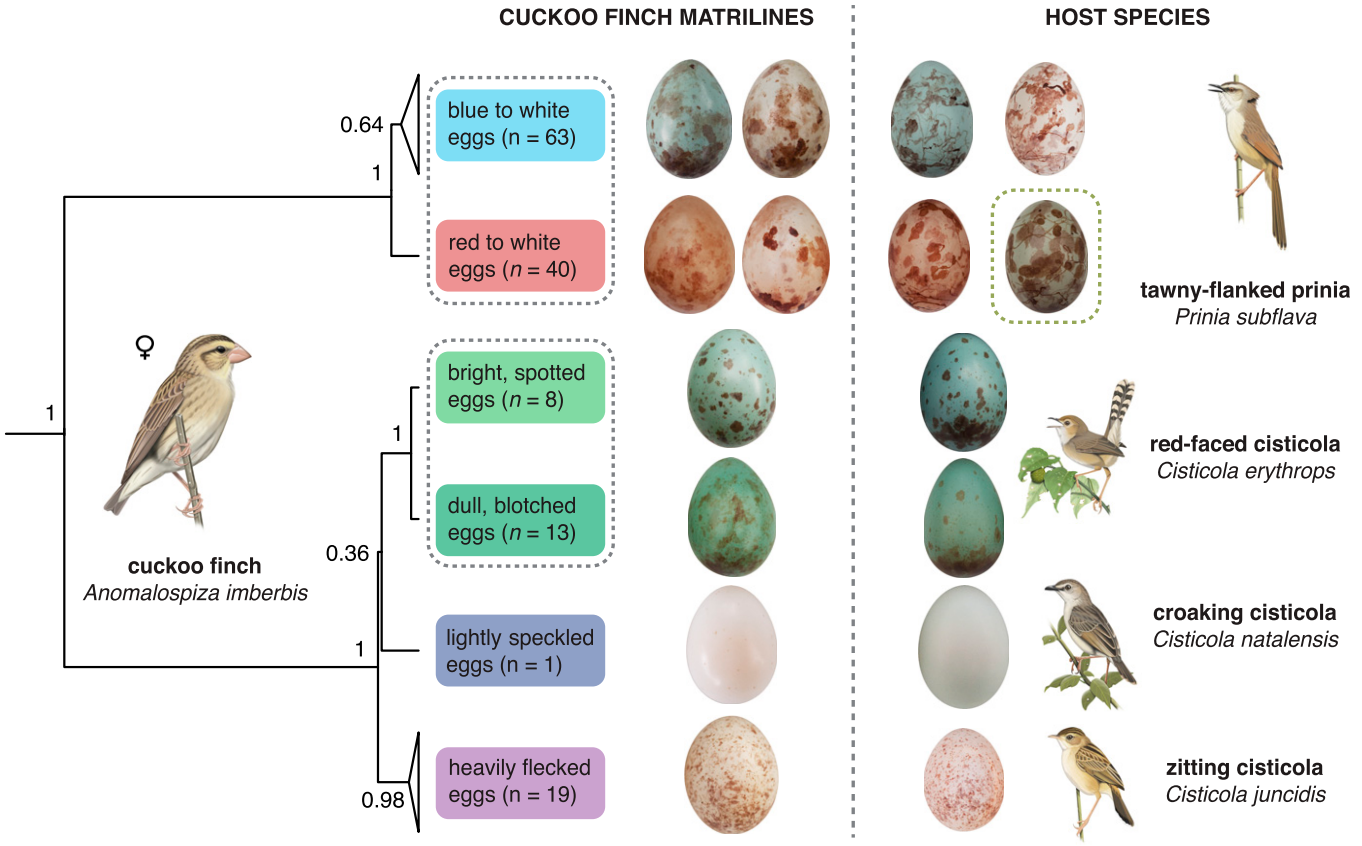

Fig. 1. Maternal inheritance of egg mimicry in cuckoo finches. Cuckoo finch females associated with different hosts and with different mimetic egg phenotypes belong to distinct mt lineages. A total of eight unique haplotypes were found for a 507-bp portion of the mt ND2 gene, including two closely related haplotypes within the blue lineage associated with *P. subflava*, and two closely related haplotypes in the lineage associated with *C. juncidis*. Within the blue and red lineages associated with *P. subflava*, the background color of cuckoo finch eggs ranged from blue to white and from red to white, respectively. At our field site, some *P. subflava* eggs have an olive-green background color (dotted box), a phenotype that is not currently mimicked by any cuckoo finches. *C. juncidis* and *C. natalensis* and the cuckoo finches parasitizing them also lay polymorphic eggs, but samples were available for only one parasitic variant for each of these host species. The mt divergence between cuckoo finches associated with *Prinia* and *Cisticola* hosts is substantial, with 9.0% uncorrected sequence divergence corresponding to an estimated divergence time of 2.3 million years (*SI Appendix*, Fig. S3). Image credit: Reprinted with permission from Chamberlain's LBJs, Faansie Peacock.

within-host variation, whereas biparental inheritance allows hosts to produce phenotypic signatures that cuckoo finches do not yet mimic.

Results

Maternal Inheritance Permits Host-Specific Egg Mimicry by Parasites. Without exception, cuckoo finches associated with *Prinia* and *Cisticola* hosts, respectively, belong to two distinct matrilines (Fig. 1), the divergence between which is extraordinarily deep, estimated at ∼2.3 million years (*SI Appendix*, Fig. S3). The perfect association of parasitic lineage with host genus indicates a lack of successful intergeneric host switches by female cuckoo finches.

Within each of these two highly divergent lineages, further matrilineal diversification tracks cuckoo finch mimicry not only of different host species, but also mimicry of different egg phenotypes within a given host species. In the cisticola cuckoo finch lineage, three distinct lineages are uniquely associated with three different host species, each with different egg coloration (Fig. 1). Cuckoo finches parasitizing red-faced cisticola are further divided into two more recently diverged matrilines, laying bright, spotted eggs, or dull, blotched eggs, respectively, corresponding to the range of phenotypes produced by their hosts (Fig. 1). Similarly, cuckoo finches parasitizing tawny-flanked prinia belong to two closely related matrilines laying eggs with different background color: one matriline lays blue or white eggs, while the other lays red or white eggs (Fig. 1). Thus, each of these prinia-specialist matrilines likely generates egg background color through differential expression of the two main pigments known from birds' eggs: biliverdin for blue or protoporphyrin for red (19), whereas eggs with white background color result when little or no pigment is deposited on those portions of the eggshell surface without pattern markings. Some tawny-flanked prinias lay olive-green eggs, but cuckoo finches do not mimic this phenotype (Fig. 1).

As expected, given the shared genealogy of mtDNA and the W-chromosome, data from 68 W-linked restriction site–associated DNA sequencing (RAD-seq) loci, obtained for females representing four of the six mtDNA lineages identified in Fig. 1, were fully consistent with the findings described in the preceding paragraph (*SI Appendix*, Fig. S4). In addition to deep divergence between cuckoo finches associated with different host genera, we found five W-chromosome variants that distinguish the more recently diverged "red" and "blue" matrilines among *Prinia*-associated cuckoo finches, and four W-chromosome variants that distinguish the lineages associated with two *Cisticola* species (*SI Appendix*, Fig. S4).

No Assortative Mating between Parasites Reared by the Same Host. A final and critical test of the maternal inheritance hypothesis is to exclude the possibility that the cuckoo finch matrilines we have characterized represent cryptic or nascent species, or perhaps partially isolated populations between which gene flow is sufficiently rare that selection on autosomal or Z-linked loci might still be effective. Based on analysis of 6,184 autosomal RAD-seq loci genotyped for 38 unrelated individuals, we detected no evidence of divergence between cuckoo finches associated with *Prinia* and *Cisticola* hosts. Autosomal Φ_{ST} for all loci combined was effectively zero (0.0021; $P = 0.071$), and BayeScan revealed no significant outlier loci (*SI Appendix*, Fig. S5). Analysis of the same data in fineRADstructure yielded no evidence of shared autosomal ancestry corresponding to host use or egg phenotype (Fig. 2). Other methods

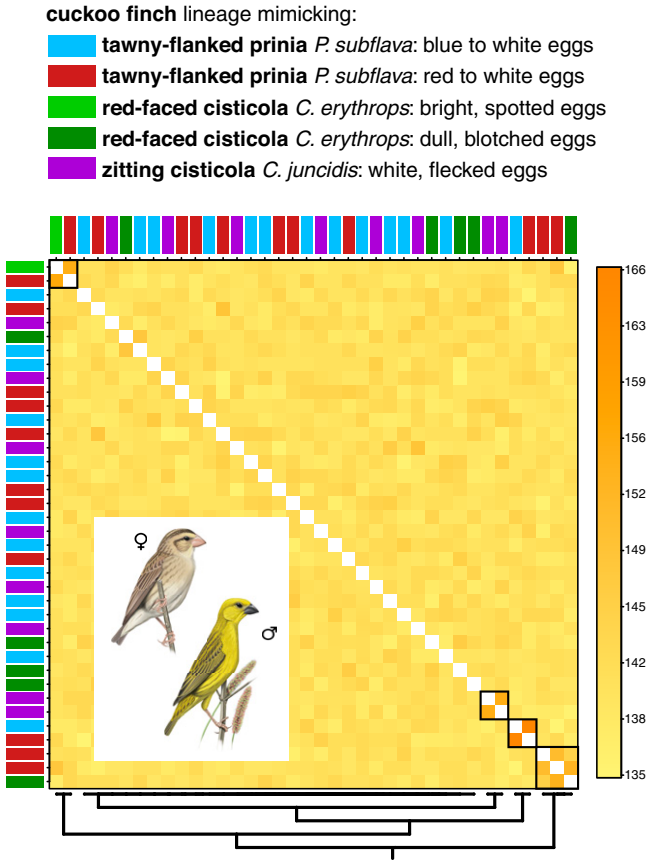

Fig. 2. Overall genomic relatedness in relation to egg mimicry matriline. Coancestry matrix from fineRADstructure (derived from 6,184 autosomal RAD-seq loci for 38 unrelated cuckoo finches) showing no evidence of genetic divergence among matrilines. Color labels indicate host species and the corresponding cuckoo finch matriline(s). Three pairs and one trio of individuals (black boxes) had somewhat higher recent coancestry, likely reflecting familial relationships more distant than half-sibs, but three of these four groups included individuals from different matrilines. These results indicate that cuckoo finch males and females raised by different hosts freely interbreed.

produced similar results (*SI Appendix*, Figs. S6 and S7), and there was no evidence of significant divergence at 282 Z-linked loci (*SI Appendix*, Fig. S8). These results confirm that all cuckoo finches at our study site belong to a single interbreeding population with no assortative mating between individuals reared by different hosts.

No Evidence of Maternal Inheritance in Tawny-Flanked Prinia Hosts. In contrast to results for cuckoo finch, there is no evidence of divergent mtDNA lineages and no association between mtDNA lineage and egg phenotype in tawny-flanked prinia (*SI Appendix*, Fig. S10), indicating that egg phenotypes in this common host species are not maternally inherited.

Discussion

Our study demonstrates that mimicry of different host-egg phenotypes is maternally inherited in cuckoo finches, which allows parasitic mothers to pass this critical host-specific adaptation to their daughters despite interbreeding with males raised by other hosts. As a result of the perfect correspondence between multiple host-specific egg phenotypes and distinct genetic matrilines, the cuckoo finch fully satisfies the expectations of Punnett's maternal inheritance hypothesis (13). We further show that maternal inheritance of egg phenotypes has not only enabled

cuckoo finches to evolve egg mimicry of different host species, as originally envisaged by Punnett, but that it also underpins their polymorphic mimicry of phenotypic variation within host species, the latter having coevolved as an escalated defense against parasitic mimicry (9, 17, 18).

The adaptive diversification of mimetic cuckoo finch egg phenotypes is remarkable because it has been accomplished in the absence of regular recombination as a source of evolutionary novelty. Given the small number of mitochondrial (mt) genes and their functions in basic cellular processes, maternal genetic control of cuckoo finch egg phenotypes almost certainly resides on the female-limited W chromosome, as envisioned by Punnett in 1933 (13). Because the entire W chromosome [except for the small pseudoautosomal region (20)] is in complete linkage disequilibrium, selection acts on the entire linkage group, which effectively includes the maternally inherited mt genome as well, rather than independently on individual loci (5). Thus, selection at any one locus is expected to be inefficient. Our results are analogous to recent evidence that the adaptive diversification of male reproductive morphs in *Poecilia parae*, a freshwater fish, involved genes residing on the nonrecombining Y chromosome (7).

In other systems, sexually antagonistic genes that are beneficial to the heterogametic sex but costly in the homogametic sex accumulate on the sex-limited (W or Y) chromosome (5, 21). In the case of cuckoo finches, a novel autosomal allele conferring better mimicry of a certain host species would differentially affect the fitness of daughters and sons. Daughters would benefit through improved mimicry of the host to which they are imprinted, whereas sons that go on to mate with one or more females specializing on another host species would pass along to his daughter an allele that reduces fitness by disrupting her mimicry of the other host. Such sexual antagonism may strengthen selection for the transfer of genes controlling egg mimicry to the W chromosome.

Unfortunately, the lack of W-chromosome recombination precludes a standard genome-wide association study (GWAS) approach to identify the specific genes involved in cuckoo finch egg phenotypes; genomic comparisons and analyses of gene expression are needed to learn more. Despite substantial variation among birds in the overall size and structure of the W chromosome, due largely to changes in repetitive, heterochromatic DNA (22, 23), most birds appear to share a conserved set of W-linked protein-coding genes (24), each of which has a Z-linked gametolog (25). However, examples of specific genes being transposed to the W chromosome (26, 27) and of neo sex chromosomes originating through the fusion of autosomes (or portions thereof) to the Z and W chromosomes in various avian lineages (28–30) highlight the possibility of adding relevant genes to the W chromosome. Alternatively or in addition, the evolution of W-linked regulation of autosomal genes (6, 21), comparable to the Y-linked polymorphisms that regulate some male-biased genes in *Drosophila* (31), may have contributed to cuckoo finch egg phenotypes; the few genes currently known to influence egg color in other birds are autosomal (32, 33), consistent with results of breeding experiments (34, 35). Once established on the W chromosome, subsequent adaptive divergence among matrilines in genes for egg appearance may have been facilitated by the action of transposable elements (TEs), as suggested for the diversification of male morphs in *Poecilia parae* (7); TEs are highly enriched on the avian W chromosome (25, 36). Thus, comparisons of gene expression (e.g., in the shell gland of laying females) and of the overall structure and gene content of the W chromosome, both between cuckoo finch maternal lineages and between cuckoo finches and related parasitic (i.e., *Vidua* spp.) and nonparasitic species (e.g., estrildid finches), may yield insights into the initial evolution of maternal inheritance as well as the subsequent diversification of mimetic phenotypes.

The lack of assortative mating with respect to host species makes maternal inheritance advantageous in cuckoo finches by allowing host specialization to evolve, but a crucial consequence of this sex linkage is the lost opportunity to recombine the traits of different cuckoo finch lineages in a single individual. Thus, while cuckoo finch matrilines associated with tawny-flanked prinia hosts have evolved eggs with either blue or red background coloration, they may be unable to express the mixture of biliverdin (blue) and protoporphyrin (red) pigments required to produce olive-green eggs that mimic those of some tawny-flanked prinia females (19, 37) (Fig. 1). By contrast, our results suggest that egg signature phenotypes in tawny-flanked prinia hosts have an autosomal genetic basis (*SI Appendix*, Fig. S10). This has likely permitted the evolution of the olive-green prinia phenotype and, in general, greater phenotypic diversity in prinias than in their specialist cuckoo finches gentes (17). In contrast to the parasite, in which the imperative is to match their respective hosts, novelty and diversity in egg phenotypes are favored in the host, such that autosomal inheritance is expected.

Further examination of how maternal inheritance constrains the evolution of egg mimicry in cuckoo finches is challenging, precisely because the majority of the W chromosome forms a single linkage group. This precludes GWAS to identity the precise genetic variants that control pigment deposition. However, a constraint associated with the lack of recombination is consistent with our limited understanding of the genetic basis of avian egg coloration. Our finding that egg coloration in the tawny-flanked prinia is autosomally inherited was expected, given that this facilitates evolution of novel phenotypes, but also because it appears to be the default condition in birds (but see ref. 38). Autosomal inheritance of egg color was demonstrated in *Gallus gallus* chickens by Punnett himself, who showed experimentally that crosses of poultry breeds with blue and red eggs, respectively, could produce birds with olive-green eggs (35). Autosomal inheritance has also been experimentally established for egg color and pattern in village weaverbirds (*Ploceus cucullatus*) (34), which have also evolved diversified egg phenotypes as a defense against parasitic cuckoos (39).

Notably, in tawny-flanked prinias there has been a recent increase in the proportion of olive-green eggs among females in our study area (40). This host phenotype should generate selection for mimicry, yet there is no evidence that any cuckoo finches produce olive-green eggs (Fig. 1) (40). Maternal inheritance and the inability to form novel combinations of existing genetic variants likely prevent cuckoo finches from evolving the ability to deposit the requisite mix of protoporphyrin and biliverdin pigments to produce this olive-green phenotype, putting it at a disadvantage at this stage of the host–parasite arms race.

Thus, while maternal inheritance ensures faithful transmission of host-specific mimicry, it requires parasites to forego recombination and heterozygosity as sources of evolutionary novelty, which may be costly in an ongoing arms race. This important theoretical limitation may apply more widely since egg polymorphisms or individual signatures within a given host species have independently evolved as a further defense against parasitic mimicry in multiple, independently evolved brood parasite-host systems (41, 42). The evolution in parasites of maternally inherited host-specific mimicry should both increase

the effectiveness of individualized egg signatures as a host defense and constrain the parasite's ability to respond, raising questions about the longer-term dynamics of these systems. For example, the linkage of beneficial combinations of host alleles might produce "unforgeable" signatures that reduce host phenotypic diversity but also accelerate parasitic switching to naïve host species. Alternatively, a parasite might become increasingly dependent on exploiting naïve host individuals who have not yet learned their own signatures and are poor rejectors of mismatched eggs (43), thus buffering both species from reciprocal selection. Such potential dynamics deserve further theoretical investigation.

In conclusion, our results suggest that at least 2 million years ago, selection from host defenses drove cuckoo finches to transfer control of egg appearance to the maternally inherited part of the genome, allowing host-specific mimicry to remain distinct in the face of gene flow between males and females raised by different hosts. In the more recent evolutionary past, this seemingly effective solution has likely limited future adaptive potential: while maternal inheritance allows cuckoo finches to exploit multiple hosts, it should slow counteradaptation to hosts that benefit from sexual recombination to evolve polymorphic egg signatures as a further defense. This likely explains why cuckoo finches have failed to "forge" certain host phenotypes, such as olive-green prinia eggs, that have an autosomal genetic basis. This is consistent with the expectation that transferring the genes underlying coevolving traits to a minor sex chromosome should effectively nullify the advantages of sexual reproduction proposed by the Red Queen hypothesis (3, 4). Thus, maternal inheritance has likely impeded, as well as facilitated, coevolution. In the case of this particular arms race, played out in grasslands of central Africa, natural selection has shaped a genetic architecture that appears to be a double-edged sword.

Materials and Methods

Study System and Sample Collection. Cuckoo finches (*Anomalospiza imberbis*) parasitize numerous species of grass-warbler in the genera *Prinia* and *Cisticola*, including four species at our study site in Zambia (a fifth host species, desert cisticola *C. aridulus*, was recorded as a host here in the 1980s but is now locally extinct). Parasitism is costly to hosts because cuckoo finch females remove host eggs when laying their own and because cuckoo finch chicks typically outcompete host chicks, causing them to starve to death (44, 45). Female cuckoo finches generally lay eggs in nests of the host species corresponding to their own mimetic egg phenotype, though they lay their eggs haphazardly with respect to variation in individual egg appearance within their host species (i.e., they do not selectively parasitize individual host females with eggs that best match their own egg phenotype) (9). Rarely, cuckoo finches lay in nests of the wrong host species; 1.8% of 276 parasitized nests at our study site during 2007–2014 contained an egg mimicking a different host species [five cuckoo finch eggs mimicking the eggs of red-faced cisticola (*C. erythrops*) were found in tawny-flanked prinia (*P. subflava*) nests, and one the other way around; these host species and their parasitic mimics have highly distinctive, nonoverlapping phenotypes (17, 46)]. In all of these cases, the parasitic eggs were rejected or deserted by hosts, suggesting that the observed error rate is an underestimate, but also that mislaid eggs are unlikely to survive.

Cuckoo finch DNA samples were collected during 2007–2009 and 2012–2014 near Choma, southern Zambia, predominantly from within approximately a 20 km^2 study area on and around Musumanene and Semahwa Farms (centered on 16°46'S, 26°54'E). The habitat is a mix of grassland, deciduous miombo woodland, active and fallow tobacco fields, and pasture. Four host species occurred syntopically within this mix of habitats, avoiding only dense woodland.

We obtained DNA samples from 196 cuckoo finches from 141 nests of the four host species (*SI Appendix*, Table S1); some host nests contained up to three cuckoo finch eggs or chicks. Almost all cuckoo finch genetic samples originated from eggs that, based on their phenotype, were laid in a nest of the expected host species. As mentioned, two samples came from eggs laid in a nest of the wrong host species; these two samples were assigned to host species based on their egg mimicry phenotype, not the identity of the host species nest in which they were found (*SI Appendix*, Table S1). Samples for genetic analysis included eggshell membranes from hatched eggs, embryos from unhatched eggs, and blood from chicks (preserved in 96% ethanol, a dimethyl sulfoxide tissue storage buffer (47), or on a Whatman FTA card). We completed double-digest restriction site–associated DNA sequencing (ddRAD-seq) for 160 individuals, and sequenced a portion of the mt ND2 gene for 145 individuals (*SI Appendix*, Table S1). The samples included in these two data sets overlapped but were not identical (*SI Appendix*, Table S1). Note that mtDNA data from a male offspring hatching from an egg of known phenotype provide complete data about his mother's matriline and phenotype, whereas W-linked loci are obtained only from females.

We tested for maternal inheritance of egg phenotypes in one host species, the tawny-flanked prinia (*P. subflava*). Using preliminary data from an in-progress study, we assembled mtDNA sequence data from whole-genome sequencing (WGS) data for 121 females (*SI Appendix*, Table S2) sampled during 2014, 2016, and 2018 from within the same 20-km^2 study area. These data were obtained from blood samples (stored on Whatman FTA cards) from females captured at their nests using mist nets and, therefore, with known egg phenotypes. The sexes in tawny-flanked prinia look alike, so females were identified in the field by wing length and the presence of a brood patch; sexing was confirmed by testing for the presence of a W-linked gene (CHD-W) in the WGS data of each female sample.

Cuckoo Finch mtDNA Sequencing and Analysis. Due to complications associated with nuclear copies of the mt genome in *Anomalospiza* (48, 49), we amplified and sequenced a portion of the ND2 gene using primers designed to selectively amplify mtDNA only (Anom.L5517mt 5′-TCACCCAAMTAACCMACCCAC-3′, Anom2.H6057 5′-CAGCTGCCATAGCCATATCCTG-3′). We sequenced PCR products using the forward primer only but carefully checked each sequence for ambiguities and confirmed each variable position by comparing all sequences in Geneious Prime 2020.0.4 (https://www.geneious.com). We omitted the first 32 base pairs (bps) of the amplified region due to ambiguities resulting from sequence compression, leaving a 507-bp region for analysis.

Among the 145 cuckoo finches we sequenced, there were eight unique mtDNA haplotypes. To provide an approximate timescale for the divergence of cuckoo finch lineages, we combined one representative of each haplotype with existing data (49–51) for the same 507-bp portion of ND2 for representatives of the other parasitic finch genus (*Vidua* spp.; $n = 10$), estrildid finches (family Estrildidae; $n = 69$) and ploceid finches (family Ploceidae; $n = 7$), and then analyzed the data in BEAST version 2.4 (52). We used a relaxed lognormal molecular clock and calibrated divergence time estimates by specifying lognormal priors on two nodes shared between our data set and the songbird diversification analysis of Moyle et al. (53). We used the following priors to match the point estimates and approximate the 95% highest posterior density intervals: 1) $\mu = 10.665$ MY and $\sigma = 0.101$ for the most recent common ancestor (MRCA) of parasitic and estrildid finches and 2) $\mu = 13.22$ MY and $\sigma = 0.074$ for the MRCA of parasitic, estrildid, and ploceid finches. The divergence times determined by Moyle et al. (39) are more recent than estimated in other studies (54, 55), making our estimates of divergence times among cuckoo finch lineages conservatively recent.

Given the relatively small data set, and based on results from jModelTest, version 2.1.5 (56), we implemented a single GTR+I+G substitution matrix for all codon positions. We completed replicate runs, each with the Markov chain run for 50 million generations, and sampled trees and parameters every 5,000 generations, excluding the first 10% of samples as burn-in. Convergence was confirmed in Tracer, version 1.7 (57), and a maximum clade credibility tree was generated using TreeAnnotator, version 2.4 (52).

ddRAD-seq and Analysis. Genomic DNA was extracted from tissue or blood samples using a DNeasy Kit (Qiagen) with 4 μL of RNase added to the lysate following incubation. RAD-seq was completed for 160 samples using the double-

digest method described in ref. 58, with pooling of samples into batches of 12 following ligation of adapters, as in ref. 59. Pooled fragment libraries were sequenced on an Illumina HiSEq 2500 in RAPID mode; 151-bp single-end reads were obtained for all samples. The sequence data were processed using a combination of custom Python scripts and publicly available software (58, 59); current versions of the code are available at https://github.com/BU-RAD-seq/Digital_RADs. We used Geneious Prime to examine and manually adjust the alignments of putative loci with five or more unique insertions or deletions (indels), two or more correlated single nucleotide polymorphisms (SNPs) in the last five bases (often indicative of misalignment due to an indel at or near the end of a locus), and/or four or more perfectly correlated SNPs at any position within the locus (typically due either to misalignment associated with an indel or the clustering of sequences from paralogous loci). End-of-locus adjustments were guided by alignments to the zebra finch *Taeniopygia gutatta* genome (60) where possible.

Given the likelihood that our data set would include many closely related individuals (e.g., full and half sibs), we first analyzed the full set of 160 samples to identify pairs and groups of close relatives. Scoring each unique full-length haplotype at a given RAD-seq locus as an allele, we identified sets of related individuals using COLONY, version 2.0.6.1 (61). We completed replicate runs, varying the priors for allelic dropout rate and other kinds of genotyping errors, from 10^{-2} to 10^{-3} to 10^{-4}. We limited these analyses to putative single-copy, autosomal loci with unambiguous genotypes for all samples, excluding all loci with missing data and those showing any evidence of paralogous loci having been clustered together (see ref. 20 for more information). This resulted in a data set of 1,386 autosomal loci, 1,215 of which included one or more polymorphisms. Excluding three samples with inconsistent recovery of loci allowed us to build a more robust data set for the remaining 157 samples, with complete data for 3,067 autosomal loci, of which 2,567 included polymorphisms. In the 160-sample analyses, each of the three excluded samples was found to be a close relative (full or half sib) with one or more other samples in the data set. After filtering out low-quality singleton reads, the median number of sequence reads for the remaining 157 samples was ∼1.15 million per sample, with all samples comprising >600,000 reads.

Results from COLONY were fully consistent, though not identical, across all runs; for example, half-sibs were arbitrarily identified as either paternal or maternal half-sibs in different runs. Results of the COLONY analyses were used to select a set of 38 unrelated individuals (i.e., less related than half-sibs), including 24 samples associated with *P. subflava* hosts, six associated with *C. erythrops*, and eight associated with *Cisticola juncidis*. This set of 38 individuals was used to test for genetic divergence between cuckoo finches associated with different hosts.

To allow for a larger number of loci in the 38-sample data set and increase our potential for detecting possible outlier loci, we included putative single-copy loci with median sequencing depth per sample ≥20 and unambiguous results for at least 36 of the 38 samples. Thus, we allowed up to two samples with missing data or low sequencing depth (i.e., <5 reads). We also excluded putative loci showing evidence of paralogous loci having been clustered together; thus, we excluded any locus with two or more heterozygous samples in which one allele accounted for <29% of reads and/or any locus with one or more heterozygous samples in which "extra" reads were inconsistent with two primary alleles/haplotypes (see ref. 58 for more details). This resulted in a data set of 6,184 autosomal loci, of which 5,210 included one or more polymorphisms (SNPs and/or indels). Each unique indel, regardless of length, was scored as a single 0/1 polymorphism. Using the same approach, we obtained a data set of 282 Z-linked loci, of which 184 included one or more polymorphisms. Z-linked loci were identified based on an ∼2:1 ratio of average sequencing depth in males versus females (*SI Appendix*, Fig. S9). The median sequencing depth for these two data sets (i.e., autosomal and Z-linked for 38 samples) was 113 reads per sample per locus, generally allowing genotypes to be scored unambiguously. Missing genotypes (0.15%), low-depth genotypes (0.15%), and heterozygous genotypes with unequal read depths (i.e., one allele accounting for <29% of reads; 0.21%) accounted for ∼0.5% of these data sets. For the latter two categories, one allele was scored as missing.

Using the set of 38 unrelated individuals, genome-wide genetic divergence was assessed using AMOVA (62), STRUCTURE, version 2.3.4 (63); fineRADstructure, version 0.3.2 (64); and principal components analysis (PCA) (65). Locus-by-locus Φ_{ST} and overall Φ_{ST} (62) were calculated using a custom Python script, comparing cuckoo finches associated with *Prinia* and *Cisticola* hosts, respectively. We randomly reassigned individuals to host species 1,000 times to a generate null distribution and test whether the observed Φ_{ST} values were significantly greater than expected under the null hypothesis of no population structure (one-tailed test). For STRUCTURE and PCA, we included multiple SNPs and/or indels per locus; analyses scoring each unique haplotype as an allele, or that randomly selected one SNP per locus, generated similar results. For PCA, we coded biallelic polymorphisms and analyzed the data in R, version 4.0.2 (66), following the approach of Novembre and Stephens (65). For PCA and STRUCTURE, we excluded rare SNPs with a global frequency <4% (i.e., three or fewer copies among the 38 individuals = 76 alleles), resulting in an autosomal data set of 9,538 biallelic polymorphisms. For PCA, missing alleles at autosomal loci (comprising ∼0.5% of the data matrix) were assigned a score equal to the allele frequency in the overall data set for that SNP or indel. For Z-linked loci, females have only one allele and were thus scored as having one observed allele and one missing allele.

For the STRUCTURE analyses, nine replicate runs were completed for each value of K (number of populations) from one through seven with 20,000 steps in the Markov chain following a burn-in of 10,000 steps. We used the admixture model with allele frequencies correlated among populations. The Evanno et al. method (67), as implemented in STRUCTURE HARVESTER, version 0.6.94 (68), inferred five distinct populations based on the autosomal data, but results for $K = 2$ through $K = 5$ were variable among runs, and none provided any evidence of samples clustering by maternal lineage (*SI Appendix*, Fig. S7).

To test for evidence of recent isolation of putative cuckoo finch host races, we analyzed the autosomal RAD-seq data using fineRADstructure, which exploits the linkage information within each locus to derive a coancestry matrix based on the most recent coalescent events (i.e., the sharing of identical or nearest-neighbor haplotypes among individuals). Based on haplotypes, this analysis effectively included all biallelic polymorphisms ($n = 16{,}097$), the most rare of which are particularly informative about recent coancestry.

Finally, to test for outlier loci showing greater than expected divergence between putative cuckoo finch host races, we analyzed the 5,210 variable autosomal loci in BayeScan, version 2.1 (69), using the default parameter settings. Given relatively small sample sizes, this analysis compared cuckoo finches associated with *Prinia* and *Cisticola* hosts, combining the more recently diverged maternal lineages associated with each host genus, respectively.

Inference of W-Chromosome Haplotypes. Given the expected maternal inheritance of both mtDNA and the W chromosome, it is reasonable to assume that mtDNA serves as a proxy for the W chromosome and that divergent mtDNA lineages are associated with comparably divergent W-chromosome lineages. To test this, we searched the ddRAD-seq data set to find W-linked loci. Typically, these were loci with data for females but not males and thus were not sequenced in a sufficient number of individuals to be included in any of the aforementioned data sets. Given a variety of possible complications, we carefully examined the data for each putative W-linked locus and manually curated the data as appropriate in Geneious Prime. For example, divergent Z-linked and W-linked gametologs (70) sometimes clustered together into a putative locus, such that females appeared heterozygous for two divergent alleles, whereas males were either homozygous or heterozygous for minimally divergent Z-linked alleles. We also found examples of duplicated or paralogous W-linked loci clustering together into a single putative locus, but we could often separate the data for each female to yield unambiguous data for two or more distinct loci. Female samples with RAD-seq data were available for four of the six cuckoo finch lineages illustrated in Fig. 1, including "prinia blue" ($n = 34$), "prinia red" ($n = 25$), "erythrops dull" ($n = 5$), and "juncidis" ($n = 16$). There was only one within-lineage polymorphism across 68 W-linked loci, so we analyzed a single consensus sequence for each lineage and locus.

Tawny-Flanked Prinia mtDNA Sequencing and Analysis. To test for maternal inheritance of egg color in one host species, we used WGS data to assemble a portion of the mt genome for *P. subflava* females with known egg phenotypes. These data are from an in-progress study on the genomics of egg color and pattern in this species. Using data derived from a tissue sample that provided substantial coverage of the mtDNA genome (∼360X), we first assembled a complete mt genome sequence using a partial *C. juncidis* sequence (GenBank accession MN356258) as an initial reference to guide the assembly. Each of the remaining

samples was then aligned to the complete *P. subflava* mtDNA using bowtie2 (version 2.3.5.1). The alignment for each sample was carefully examined in Geneious (version 2021.2.2) to confirm all variant sites. This was necessary because 151 of 155 *Prinia* DNA extracts were from blood samples, which yield fewer mtDNA sequences (mature red blood cells in birds lack mitochondria) and, as in many other birds, also yield more or less divergent nuclear copies of portions of the mt genome (58). Because of complications associated with nuclear copies, we restricted our analysis to a 2,245-bp region comprising the ND1 and ND2 genes along with three intervening transfer RNA genes. In contrast to other parts of the mt genome, nuclear copies of this region were evident in only a fraction of samples (~14%), and when they occurred, they could be unambiguously recognized and filtered out in most cases. mtDNA genome coverage for the 151 blood samples ranged from ~7X to ~40X; samples with less than ~11X coverage often included small gaps with little or no coverage. Excluding these lower-coverage samples ($n = 16$) and several others that included one or two "polymorphic" sites for which the mt state could not be determined with certainty ($n = 10$) resulted in a final data set of 129 females. The 26 excluded samples included the full range of egg phenotypes, and there was no evidence of divergent mt haplotypes in the excluded set. Given minimal divergence among haplotypes, we summarized the results in a simple parsimony network.

Data Availability. Genetic data from this study have been deposited in GenBank, including cuckoo finch ddRAD-seq data (PRJNA809323) (71), cuckoo finch mtDNA data (OM791460:OM791604) (72) and tawny-flanked prinia mtDNA data (OM858881:OM859001) (73). Cuckoo finch egg color spectra are deposited in Dryad (DOI: 10.5061/dryad.rn8pk0pck) (74).

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