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Apparent vector-mediated parent-to-offspring transmission in an avian malaria-like parasite

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Abstract

Parasite transmission strategies strongly impact host–parasite co-evolution and virulence. However, studies of vector-borne parasites such as avian malaria have neglected the potential effects of host relatedness on the exchange of parasites. To test whether extended parental care in the presence of vectors increases the probability of transmission from parents to offspring, we used high-throughput sequencing to develop microsatellites for malaria-like *Leucocytozoon* parasites of a wild raptor population. We show that host siblings carry genetically more similar parasites than unrelated chicks both within and across years. Moreover, chicks of mothers of the same plumage morph carried more similar parasites than nestlings whose mothers were of different morphs, consistent with matrilineal transmission of morph-specific parasite strains. Ours is the first evidence of an association between host relatedness and parasite genetic similarity, consistent with vector-mediated parent-to-offspring transmission. The conditions for such 'quasi-vertical' transmission may be common and could suppress the evolution of pathogen virulence.

Keywords: avian blood parasites, high-throughput sequencing, horizontal and vertical transmission, *Leucocytozoon*, local transmission, vector

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Introduction

Parasitism is a fundamental driver of organismal evolution (Hamilton *et al.* 1990; Bergstrom & Antia 2006). Theoretical models suggest that parasite virulence is determined by the ratio of horizontal to vertical transmission and the spatial and demographic structuring of host populations (Frank 1996; Boots *et al.* 2004; Lion & Boots 2010). Transmission by vectors is usually considered to be horizontal within a population, implying that it is random with respect to host family identity/kinship and therefore occurs mainly between unrelated hosts. However, many behavioural and life history traits of hosts, such as site fidelity and family cohesion, could potentially enhance the potential for transmission between parents and offspring or otherwise related individuals via vectors, with important implications for parasite virulence (Clayton & Tompkins 1994; Lion & Boots 2010). Such transmission could be termed 'quasi-vertical' as it differs substantially from both horizontal and vertical (e.g. mother to embryo) transmission (Fig. 1). Similarly to vertical transmission, quasi-vertical transmission may limit the potential virulence of parasites.

Malaria-like parasites are a large and highly diverse group that is attracting increasing attention (Bensch *et al.* 2009). However, population genetic studies are largely restricted to a handful of *Plasmodium* species of humans and one lizard species (Awadalla *et al.* 2001; Schall & Vardo 2007), which have atypically low host specificity, high virulence and reproduce asexually within blood cells (Valkiūnas 2005). Characterizing the population structure and dynamics of blood parasites outside of the genus *Plasmodium* is therefore necessary for understanding blood parasite evolution as a whole.

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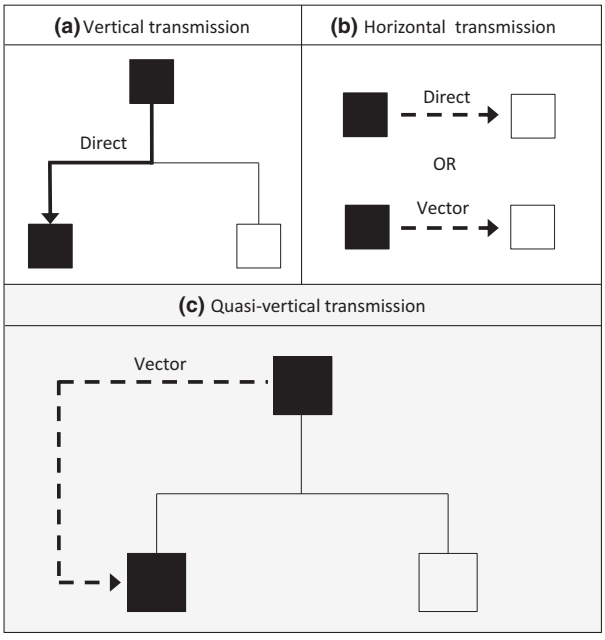

Fig. 1 Modes of transmission. (a) Vertical transmission of parasites takes place from a parent to an unborn or newborn offspring directly, that is without a vector (Busenberg & Cooke 1993). (b) Horizontal transmission is dependent on geographic distance but assumes no relatedness between hosts. (c) Quasi-vertical transmission takes into account the correlation of relatedness and geographic distance between individuals. It occurs between relatives such as parent and offspring via vectors. Black and white squares indicate infected and uninfected hosts, respectively. Arrows show transmission, with dashed lines indicating geographic distance-dependent transmission.

In particular, members of one major group of vector-borne pathogens, avian malaria-like blood parasites of the genus *Leucocytozoon*, have been suggested to utilize the long altricial periods of their hosts for transmission (Ashford *et al.* 1991; Valkiūnas 2005). *Leucocytozoon* are transmitted by day-active blackflies, which breed in streams and can transmit the infection several days after biting an infected host (Valkiūnas 2005).

Host groups such as diurnal birds of prey offer vectors a prolonged period of access to chicks, which could increase the potential for parasite transmission. This leads to the expectation that parasites of raptors could be impacted by host family structure. Additionally, many raptor species have plumage polymorphisms, which appear to be genetically determined (Krüger 2008). In common buzzards, *Buteo buteo*, assortative mating with respect to plumage morph leads to strong demographic structuring of the host population (Krüger *et al.* 2001; Boerner & Krüger 2009) and the morphs also differ in their parasitism rates (Chakarov *et al.* 2008). This could potentially lead to morph-specific parasite compositions, especially for vertically or quasi-vertically transmitted parasites.

Until recently, a major obstacle to understanding the transmission and population dynamics of nonmodel pathogens was the development of genetic markers. For avian blood parasites, universal primers are available for a few genes (Hellgren *et al.* 2004; Bensch *et al.* 2009), but markers with sufficient polymorphism to explore fine-scale parasite population structure are lacking. High-throughput sequencing approaches capable of generating billions of bases in a single run provide a solution. However, with only a tiny percentage of nucleated cells being infected (<5%) and the parasite genome being approximately 150 times smaller than that of the host (approximately 20 Mbp versus approximately 3 Gbp, respectively), shotgun sequencing of blood samples would be expected to generate negligible amounts of parasite sequence data. Fortunately, recent developments allow the enrichment of parasite cells (Henry & Dick 1978; Omori *et al.* 2010; Palinauskas *et al.* 2010, 2013; Chakarov *et al.* 2012), making it possible to obtain nuclear genomic parasite sequences from which rapidly evolving markers such as microsatellites may be developed. Microsatellites are particularly useful due to their high levels of polymorphism and can be successfully PCR amplified from blood extracts even when parasite DNA concentrations are low.

Here, we used high-throughput sequencing to develop microsatellites for avian *Leucocytozoon* parasites. These were then genotyped in a natural population of common buzzards to analyse parasite population structure. We predicted that, under quasi-vertical transmission, (i) closely related buzzard chicks should carry more genetically similar *Leucocytozoon* parasites than unrelated chicks, and (ii) *Leucocytozoon* strains should be more similar within than among plumage morphs as morph is one of the major demographically structuring traits in common buzzards, potentially leading to the transmission of characteristic clone compositions.

Materials and methods

Study population and blood samples

The study was performed on a natural population of common buzzards extending over an area of approximately 300 km^2 in eastern Westphalia, Germany, which has been intensively studied for the last 24 years. Common buzzards (*B. buteo*) are the most common bird of prey and the only known host of the parasite *L. buteonis* in central Europe (Valkiūnas *et al.* 2010). Buzzards produce a single brood per year between March and May consisting of one to four chicks. As in many other raptors, pair bonding usually lasts until one of the partners dies and extra-pair paternities are presumed to be very infrequent (e.g. Gavin *et al.* 1998).

Each year, all woods in the study area were searched for active nests and these nests were repeatedly visited until the identities and morphs of breeding adults and expected hatching dates of their broods were established. In the study area, the density of breeding buzzard pairs varies annually between 10 and 60 pairs per 100 km^2 (Chakarov & Krüger 2010) and the mean nearest neighbour distance, which in other populations has been shown to correspond to adult home ranges, is approximately 830 m (Walls & Kenward 2001). Nests can be reused between years, but new nests are often built, usually within 200 m of old nests in the same territory (Krüger 2002).

When the chicks were approximately 20 days old, trees containing active nests were climbed. All chicks were lowered to the ground, ringed and weighed, left tarsus length was measured, and morph was recorded. We distinguished three plumage morphs termed 'dark', 'intermediate' and 'light', which show simple Mendelian inheritance, light and dark individuals being putatively homozygous for one of two alleles and intermediately melanized birds being heterozygous (Krüger *et al.* 2001). Dark birds have a dark head, heavily speckled or dark breast and underwing coverts. Intermediate individuals have a dark head, intermediately speckled breast and underwing coverts. Birds with little to no breast and underwing coverts melanization, in extreme cases with light head and upperwing coverts, belong to the light buzzard morph (Chakarov *et al.* 2013). The colour morphs are not spatially segregated within the study area. A blood sample of 50–500 μL was taken with a syringe from the brachial vein of each chick, a blood smear was prepared, and the rest of the sample was stored in ethanol or PBS-EDTA buffer. The chicks were then returned to their nests. All field procedures were conducted with the permission of the local authority Kreis Gütersloh (permit nr: 4.5.2-723-Bussard).

Mitochondrial sequencing

Infected buzzard chicks were identified through inspection on Giemsa-stained blood smears (Chakarov *et al.* 2008). DNA was extracted from the *Leucocytozoon*-infected samples using a standard phenol–chloroform protocol. To analyse the composition of *L. buteonis* mitochondrial lineages in our buzzard population, we sequenced 700 bp of the cytochrome oxidase subunit III (COXIII) for 80 individual infections. We performed PCR reactions with 40 μM primers LbutCOIIIF (GGAAACACACTTCCCTTCTCGCCA) and LbutCOIIIR (GCGTGACGAGCGGTGTGTACA), 2 mM Mg^{2+}, 40 μM dNTPs, PCR buffer and 1 U Taq (5 PRIME, CatNr 2200020, following the 5 PRIME Taq protocol) and 20–200 ng DNA from infected buzzard blood under 35 cycles at an annealing temperature of 57 °C. The resulting products were purified with exonuclease and shrimp alkaline phosphatase and then cycle-sequenced in both directions using the forward and reverse primers and ABI BigDye Terminator. Sequencing products were cleaned with Sephadex columns and resolved on an ABI 3730 Analyser.

Parasite enrichment

The primary aim of this study was to develop fast-evolving genetic markers for *Leucocytozoon* to evaluate the degree of support for the hypothesis of quasi-vertical transmission. However, this study also provided the opportunity to generate genomic data for both the parasite and host. Rather than isolating parasitic and avian cells for separate sequencing of their genomes (Palinauskas *et al.* 2013), we therefore opted to enrich for infected cells in raw infected buzzard blood, allowing us to partially sequence the 'dual genomes' of *Buteo* and *Leucocytozoon*. The enrichment was based on density gradient centrifugation (Henry & Dick 1978) which makes use of the greater size and lower density of cells infected with *Leucocytozoon* compared to uninfected erythrocytes. Briefly, within several days of initial sampling, chicks with high infection intensities (3–5% of all erythrocytes infected by *Leucocytozoon*, as immediately visible on Giemsa-stained blood smears even before prevalence estimation) were resampled. Five hundred microlitre blood was taken into heparinized capillaries and transported to the laboratory on ice. Sterile stock isotonic Percoll solution (SIP, Amersham Biosciences) was then manually layered in 15-ml Falcon tubes with bottom to top: 5% of tube volume filled with 45% SIP (diluted in 0.15 M NaCl solution), 10% filled with 35% SIP, 20% with 30% SIP, 15% with 22.5% SIP and 15% with 15% SIP. The raw infected blood was carefully layered on top of the Percoll gradient and centrifuged (20 min, 14 000 ***g***). Each layer was then isolated, and small subsamples were immediately inspected under a light microscope. Layers containing many *Leucocytozoon*-infected cells but also other cells were subjected to additional rounds of density gradient centrifugation (Henry & Dick 1978). After several trials, samples of two buzzard chicks could be enriched to a perceived infection rate of 80–95% of all cells.

High-throughput sequencing, assembly and microsatellite development

Library preparation and Illumina HiSeq 2000 sequencing were carried out by Harvard FAS Center for Systems Biology (Cambridge, USA) using standard

protocols. The resulting 100-bp paired-end reads were assembled using the overlap consensus assembler Newbler 2.7 (454 Life Sciences) using the default filter built into Newbler. Of a total of 1.212 billion reads, 1.116 billion passed the filter, resulting in an assembly of 39 346 scaffolds totalling 1.176 GB, with a peak coverage of 89-fold. To assign contigs to either parasite or host, we BLASTed all contigs against the human malaria *Plasmodium falciparum* and chicken *Gallus gallus* genomes using an e-value threshold of e^{-10}. Sequences revealing homology to *Plasmodium* and longer than 20 kb were used to develop *Leucocytozoon* microsatellites (Martins *et al.* 2009). Only putative microsatellite loci with at least six tri- to hexanucleotide repeats were selected for development, as these tend to give clearer banding patterns compared to dinucleotide repeats. We identified 36 tri-, seven tetra-, two penta- and one hexanucleotide repeat (see Table S1, Supporting information for details). Primers were designed for all of these loci using PRIMER 3 (Martins *et al.* 2009; Untergasser *et al.* 2012). These were then tested for amplification in nine infected buzzards and two negative controls. PCR amplification was undertaken with universal FAM-labelled M13 primers using a Type-it microsatellite PCR kit (QIAGEN). The resulting fragments were resolved on an ABI 3730 Automated DNA Analyser and analysed using GENEMARKER 1.95 (SoftGenetics LCC).

Microsatellite data set and statistical analyses

All of the microsatellites yielded clearly interpretable PCR products and were polymorphic in the nine infected buzzards but did not amplify in the negative controls. We therefore selected 15 loci with the clearest banding patterns for incorporation into two multiplexes (Holleley & Geerts 2009) (Table S1, Supporting information). These loci were then genotyped in 126 infected chicks sampled between 2009 and 2013 and belonging to 92 broods of 61 adult female buzzards. This amounted to infections of 42 broodmate pairs, 89 pairs of siblings raised in different years and 7744 pairs of unrelated chicks. Of the chicks, 16 were of the dark morph, 58 were intermediate and 52 were light. Among the 61 mothers whose offspring were included, six were of the dark morph, 34 were intermediate and 15 were of the light morph. In six cases, the morph of the mother could not be determined.

Clonality of *Leucocytozoon* parasites was estimated through multilocus linkage disequilibrium, measured with the standardized index of association (I_A^S) (Anderson *et al.* 2000a), calculated with the R package poppr (Kamvar *et al.* 2014). This index estimates the tendency towards associations of alleles between different loci (i.e. not full recombination of alleles as could be expected for tentatively noninterbreeding clones). We estimated parasite genetic similarity between chick *Leucocytozoon* infrapopulations based on the 15 microsatellite loci using the function meandistance.matrix in the package polysat in R 3.1.1 (Bruvo *et al.* 2004; Clark & Jasieniuk 2011). This package is designed to analyse data sets with variable levels of ploidy among loci and individual samples, resembling the pattern of multiple coinfecting genotypes. Genetic similarity was calculated as one minus the genetic distance between two parasite populations, computed for each microsatellite locus and averaged across all of the loci (Bruvo *et al.* 2004). To determine the factors that affect parasite composition, we constructed a series of generalized linear mixed models (GLMMs) of parasite genetic distance with the R package lme4. The identities of both individuals compared and the broods they belonged to were fitted as random factors, and a Gaussian error structure was specified. The predictor variables fitted were (i) chick relatedness, as a categorical variable with levels 'unrelated', 'siblings from different years' and 'nestmates' (i.e. siblings from the same year); (ii) morph of the compared chicks, as a categorical variable with levels 'same' and 'different'; (iii) mother's morph, specified as above; (iv) father's morph, also specified as above; (vi) linear geographic distance between the chicks; and (vii) the time difference in years between the hatching dates of the chicks. The statistical significance of each predictor variable was determined by term deletion and comparison of models with likelihood ratio tests (LRTs). The significance of differences between factor levels was assessed from final models including only significant predictors of genetic similarity with LMERConvenienceFunctions. To control for any nonindependence within the data, we also performed partial Mantel tests with 10^6 permutations using matrices of the dependent variable, parasite similarity, and the explanatory variables chick relatedness, geographic distance between chicks, time difference between chicks, whether or not the chicks were of the same morph, whether or not the chick's mothers were of the same morph and whether or not the chick's fathers were of the same morph. Partial Mantel tests were implemented within the R package ecodist (Goslee & Urban 2007).

Results

Mitochondrial lineage diversity, assembly and clonality

During our study, the annual *Leucocytozoon* prevalence among buzzard chicks varied between 33% and 52% with an overall mean of 45% (N. Chakarov, M. Boerner, O. Krüger, unpublished). Sanger sequencing of a 700-bp COIII fragment in 80 infections did not reveal any poly-

morphisms, indicating the presence of a single mitochondrial *L. buteonis* lineage in the host population (GenBank Accession no.: KP640617, also available in Dryad). We therefore used high-throughput DNA sequencing of *Leucocytozoon*-enriched common buzzard blood to derive a panel of hypervariable parasite-specific microsatellites. The dual genome assembly based on 1.116 billion reads yielded 908 463 contigs with n50 = 36,406 bp. Plotting contig length against the number of reads assembled into each contig revealed two clusters of approximately 80× and 30x (31–36) average coverage, respectively (data not shown). BLASTing these contigs against the chicken (*G. gallus*) and human malaria (*P. falciparum*) genomes, the higher-coverage contigs were found to comprise mainly vertebrate (i.e. buzzard) sequences. Among the contigs with lower coverage, 154 were >20 kb in length and revealed significant similarity to *Plasmodium* sequences. These putative *Leucocytozoon* contigs had a cumulative length of 4.11 Kbp, corresponding to 15–17% of the genome size of known *Plasmodium* species (23–26 Mbp). Within 37 of these contigs, we identified 46 microsatellites, all of which were polymorphic when tested in blood DNA extracts of nine infected buzzards and failed to amplify in two uninfected buzzard negative controls [Table S1 (Supporting information), microsatellites with flanking sequences are deposited in Dryad]. Fifteen of these *Leucocytozoon* microsatellites were selected for screening in our study population. Because haemosporidian gametocytes are haploid in vertebrate hosts, the presence of multiple alleles at a given locus within a single host indicates infection with multiple parasite genotypes. Each buzzard individual carried on average two alleles per locus, indicating that mixed infections are common in the host population. Highly significant linkage disequilibrium was found among the loci ($I_A^S = 0.051$, $P < 0.0001$), indicating clonal structure of *Leucocytozoon* infections and supporting the interpretation of different alleles as clones (Anderson *et al*. 2000a). For the most diverse loci, the number of alleles per individual, typically interpreted as the number of clones in an infection (Anderson *et al*. 2000b; Ferreira *et al*. 2007), was three on average and reached up to 10.

Genetic relatedness of parasite infections by host relatedness and morph

The genetic similarity of *Leucocytozoon* compositions, quantified using 15 microsatellite loci, was lowest among unrelated chicks. Full-siblings, both within and between years, had significantly higher levels of parasite similarity than unrelated chicks (Table 1, Fig. 2, LRT, $\chi^2_{2,7875} = 17.217$, $P < 0.001$). To control for any nonindependence in the data, we also analysed matrices of the dependent and independent variables within a partial Mantel test framework. Chick relatedness remained highly significant (R = 0.053, $P < 0.001$) even after controlling for geographic distance, the time difference between samples, and whether or not the chicks and their mothers and fathers were of the same morph. When fitted alone in a model of parasite genetic similarity, the geographic distance between chicks was found to be weakly significant (LRT, $\chi^2_{1,7875} = 6.032$, $P = 0.014$). However, geographic distance was not a significant predictor of parasite similarity when only pairs of unrelated chicks were fitted in the model (LRT, $\chi^2_{1,7744} = 2.246$, $P = 0.134$). This makes sense as full-siblings, both within and between years, are geographically very close together and have elevated parasite similarity. A partial Mantel test also did not show a significant effect of geographic distance on parasite similarity (R = −0.010, $P = 0.635$).

Parasite similarity of unrelated chicks was not explained by the nestlings having the same morph (LRT, $\chi^2_{1,\ 7744}=0.006$, $P = 0.936$, partial Mantel test R = 0.013, $P = 0.227$) or by their fathers having the same morph (LRT, $\chi^2_{1,6211}=0.852$, $P = 0.356$, partial Mantel test R = -0.028, $P = 0.917$). However, offspring of mothers belonging to the same plumage morph carried significantly more similar parasite clones than chicks whose mothers were of different morphs (Fig. 3; LRT, $\chi^2_{1,6774}$, =14.517, $P < 0.001$, partial Mantel test R = 0.134, $P < 0.001$). This is consistent with potential morph-specific *Leucocytozoon* strains being transmitted mostly from mothers to offspring.

Discussion

Within the host populations, vector-borne pathogens are generally assumed to be transmitted randomly with respect to host relatedness. In contrast, we found that common buzzard siblings carry parasites with higher genetic relatedness than unrelated chicks. Such a correlation between endoparasite genetic similarity and host family structure may arise through siblings receiving similar parasite clones from the same donors. Additionally, the genetic similarity of parasites residing within unrelated hosts is explicable by a population-structuring trait of their most probable donors, that is the plumage morph of their mothers.

High parasite genetic similarity between nestmates could potentially result from horizontal transmission. However, elevated parasite relatedness between siblings hatched in different years cannot invoke horizontal transmission between these individuals that have never been in close spatial proximity. This is because natal philopatry is rare in buzzards and is especially unlikely to occur while territories are still occupied by the parents (Walls & Kenward 2001).

Table 1 Outputs of models of genetic similarity of *Leucocytozoon* parasites. Chi-squared values and *P*-values were derived through likelihood ratio tests following term deletion (see Materials and methods for details). In the first model, (a) we analysed the contributions of chick relatedness, fitted as a three-level factor, together with geographic distance and the time difference between the chicks compared (n = 7875 dyads, 126 nestlings). In subsequent models, we tested whether parasite similarity was significantly greater in unrelated (b) chicks of the same morph relative to chicks of a different morph (n = 7744 dyads); (c) chicks whose fathers were of the same morph versus chicks whose fathers were of a different morph (n = 6211 dyads); and (d) chicks whose mothers were of the same morph versus chicks whose mothers were of a different morph (n = 6774 dyads)

	Estimate	SE	t-value	χ^2	*P*
(a)					
Intercept	0.505	0.008	62.027		
Chick					
Unrelated versus siblings from different years	0.019	0.011	1.690	17.217	0.0002
Unrelated versus nestmates	0.062	0.016	3.849		
Geographic distance	−0.001	0.000	−1.431	2.049	0.1523
Difference in years between chicks	−0.006	0.003	−2.280	5.006	0.0253
(b)					
Intercept	0.499	0.008	65.110		
Chicks have same morph	0.000	0.002	−0.080	0.006	0.9365
(c)					
Intercept	0.500	0.008	59.077		
Chick's fathers have same morph	−0.003	0.003	−0.922	0.852	0.3560
(d)					
Intercept	0.497	0.008	60.852		
Chick's mothers have same morph	0.011	0.003	3.817	14.517	0.0001

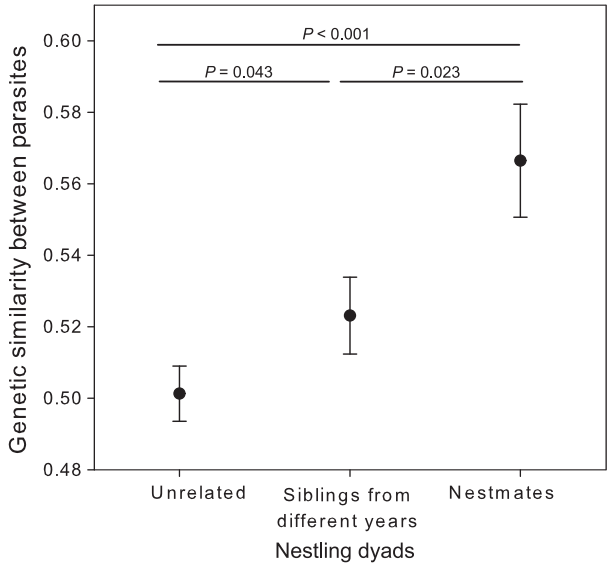

Fig. 2 Genetic similarity between parasites of siblings and unrelated chicks. Genetic similarity (estimates ± SE) between *Leucocytozoon buteonis* parasites of common buzzard chicks, based on 15 microsatellite loci. Compared groups are nestmates (i.e. full-siblings raised together within a single nest), full-siblings from different years (which were raised by their genetic parents but did not encounter one another) and unrelated chicks. Estimates are obtained from the final mixed effects models, containing only significant predictors.

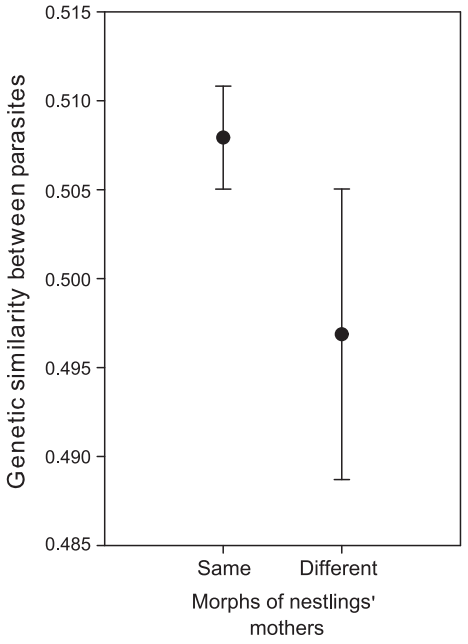

Fig. 3 Genetic similarity between *Leucocytozoon* parasites of nestlings depending on the plumage morphs of the mothers. Genetic similarity (estimates ± SE obtained from the mixed effects model) between *L. buteonis* infections of common buzzard chicks belonging to mothers of identical or different plumage morphs. Sibling dyads are not included.

Whether quasi-vertical transmission arises for vector-transmitted diseases will depend on the behaviour of the specific vectors. The high survival and fitness costs to vectors of ingesting infected blood may substantially reduce the radius of transmission (Waite *et al.* 2012, 2014; Lalubin *et al.* 2014; Levin & Parker 2014). *Leucocytozoon* infections cannot be transmitted over long periods of time by their blackfly (*Simuliidae*) vectors because adult blackflies are short-lived and do not survive over winter (Crosskey 1990). Compared with other insect vectors, blackflies can cover great distances, although some species show strong species specificity (Hellgren *et al.* 2008) and a great majority of individuals may tend to remain around a stable food source (Crosskey 1990; D. Werner pers. communication). As *L. buteonis* is a buzzard-specific lineage, the most parsimonious explanation for our findings is that parasite similarity reflects shared donors, most likely the chick's parents. Compared to many other avian hosts, the breeding density of buzzards is low and distances between occupied nests are large, which could lead to local transmission occurring mainly between parents and offspring. Thus, we did not find a significant effect of distance between nests above and beyond that of the immediate family (but see N. Chakarov, M. Boerner, O. Krüger, in preparation for spatial patterns of *Leucocytozoon* prevalence). The strength of quasi-vertical transmission may also be expected to be strongly influenced by the density of vector-breeding sites such as streams which is high in our study area (Wood *et al.* 2007; Knowles *et al.* 2011).

A second line of support for parasite-mediated vertical transmission is provided by the observation that maternal but not paternal or chick plumage morph is significantly associated with chick parasite similarity. We previously showed that the three buzzard morphs differ in *Leucocytozoon* prevalence (Chakarov *et al.* 2008), but our current study suggests that they may also differ in parasite composition. To unequivocally demonstrate quasi-vertical transmission would require the sampling of both adults and their young at the same time. Unfortunately, this was not possible in our study due to the difficulty of sampling adult raptors. However, the effect of maternal morph on parasite composition is consistent with quasi-vertical transmission because buzzard mothers remain at their nests while the fathers spend most of their time searching for prey items (Glutz von Blotzheim *et al.* 1971). This makes the mother by far the more probable parasite donor. It therefore seems feasible that female buzzards harbour and pass down parasites that are more similar in mothers of the same morph (Fig. 3).

It remains to be seen whether clone compositions are affected by the immunity of female buzzards, although in mice the strength and immediacy of the immune response do not affect *Plasmodium* clone composition (Grech *et al.* 2008). Morph-specific immunity could potentially shape the parasite community of mothers during their lifetimes, thereby influencing the parasite diversity passed onto the next generation (Galeotti & Sacchi 2003; Gasparini *et al.* 2009; Gangoso *et al.* 2011). Moreover, the immune response might even mask patterns resulting from quasi-vertical transmission if parental immunity enforces a turnover of parasite clones between successive breeding attempts (Bruce *et al.* 2000). Thus, depending on the system, quasi-vertical transmission could lead either to enhanced parasite similarity or to negative frequency dependence of clones between sibling batches. Studying the interaction between quasi-vertical transmission and immunity therefore presents a promising area for future research.

Avian blood parasites have been intensively studied over the past two decades at the level of mitochondrial lineages, and high levels of parasite diversity are often observed within host species, especially outside of the genus *Plasmodium* (Bensch *et al.* 2009). However, we found no parasite mitochondrial sequence variation within our study population, necessitating the development of polymorphic nuclear markers via high-throughput sequencing. The latter approach revealed high levels of microsatellite diversity, with up to 17 distinct alleles per locus indicative of at least as many *L. buteonis* clones being present in the buzzard population as a whole and up to 10 clones being present within a single host individual. This remarkable level of diversity in the absence of distinct mitochondrial lineages suggests that, more generally, avian malarial parasite diversity could be higher than previously envisaged. Such diversity may arise from host population structuring and distinct transmission routes, which in turn depend on the ecology of hosts and vectors.

Our study is suggestive of quasi-vertical transmission of malaria-like parasites in the common buzzard. The necessary conditions for quasi-vertical transmission – extended parental care, territoriality, familial aggregations or any other cause for family cohesion in space – are reasonably common. It is therefore possible that this mode of transmission could be widespread among directly and vector-transmitted parasites and symbionts. Parasite population structure in turn holds the potential to drive the co-evolution of host and parasite demes and may lead to predominantly commensal or pathogenic host–parasite relationships (Clayton & Tompkins 1994; Dybdahl & Storfer 2003). Certain parasites may therefore affect fitness even more strongly than tends to be assumed based on prevalence and intensity, although this will depend critically on the timescale over which family cohesion persists and the developmental window of both the pathogen and vector.

Parasite population genetic studies are often hampered by the laborious development of genetic markers, which has largely been justifiable only for parasites of humans and economically relevant species. This has severely curtailed our understanding of the diversity of short-term transmission and coadaptation processes, which form the basis of deeper evolutionary patterns (Ricklefs & Fallon 2002). As the present study demonstrates, new sequencing platforms greatly facilitate the exploration of nonmodel parasite life histories. In particular, our data are suggestive of a transmission strategy that, although largely neglected, may be common and could greatly influence host–parasite co-evolution, virulence and host fitness.

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N.C. conceived the idea. N.C., M.B. and O.K. performed sample collection and parasite cell enrichment. B.L. and A.G. participated in genome assembly. N.C., M.B., J.I.H. and O.K. carried out infection genotyping and data analyses. N.C., J.I.H. and O.K. wrote the manuscript.

Data accessibility

Microsatellite loci developed for *Leucocytozoon buteonis* have been listed together with their primer sequences in Table S1 (Supporting information). Data sets of genotypes, genetic distances, data on the buzzards, developed microsatellites and flanking sequences and the code used for the statistical analyses have been deposited under http://dx.doi.org/10.5061/dryad.s597d. Mitochondrial COIII sequence has been deposited under GenBank Accession no. KP640617.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Microsatellites for genotyping of *L. buteonis*. Microsatellite identification and primer design are based on a dual genome assembly of *Buteo–Leucocytozoon*, where only repeat sizes longer than two base pairs are included (see main text). Primers with the indicated fluorescent dyes and allele size distributions were used for parasite genotyping.