A quantitative trait locus for recognition of foreign eggs in the host of a brood parasite

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Abstract

Avian brood parasites reduce the reproductive output of their hosts and thereby select for defence mechanisms such as ejection of parasitic eggs. Such defence mechanisms simultaneously select for counter-defences in brood parasites, causing a coevolutionary arms race. Although coevolutionary models assume that defences and counter-defences are genetically influenced, this has never been demonstrated for brood parasites. Here, we give strong evidence for genetic differences between ejector and nonejectors, which could allow the study of such host defence at the genetic level, as well as studies of maintenance of genetic variation in defences. Briefly, we found that magpies, that are the main host of the great spotted cuckoo in Europe, have alleles of one microsatellite locus (Ase64) that segregate between accepters and rejecters of experimental parasitic eggs. Furthermore, differences in ejection rate among host populations exploited by the brood parasite covaried significantly with the genetic distance for this locus.

Introduction

Coevolution is a widespread process in nature that, for instance, is believed to be partly responsible for biodiversity (Thompson, 1999). Therefore, understanding the coevolutionary process is of great importance in evolutionary biology. Avian brood parasitism is one of the most well studied model systems of coevolutionary interactions, and it has been proposed as a model system for studying coevolutionary processes (Rothstein, 1990). This is because avian brood parasites exert strong selection pressures on their hosts and defensive and counter-defensive mechanisms (e.g. foreign egg recognition and rejection by host and egg mimicry by parasites) are easily detected by humans (Rothstein, 1990). Once host defensive phenotypes spread in the population, counter-defensive mechanisms in brood parasites are of a selective advantage, giving rise to a coevolutionary arms race between host and parasite (e.g. Davies, 2000).

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Cultural co-evolution cannot occur, then a prime condition is a genetic basis for defences and counter-defences otherwise evolution will not occur (Fisher, 1930). However, although some evidence is consistent with a genetic basis of host (Rothstein, 1975, 1982; Briskie *et al.*, 1992; Soler *et al.*, 1999a) and parasite behaviour (Marchetti *et al.*, 1998; Gibbs *et al.*, 2000; Payne *et al.*, 2002), genetic control and its extent has not been directly studied. Thus, since all models assume a genetic basis of traits involved in the process (see e.g. Takasu, 1998; Robert *et al.*, 1999; Servedio & Lander, 2003), a study of the genetic determinants of anti-parasite behaviour by hosts is essential for testing a critical assumption of theory. Thus, if a marker of, for instance, the genetic capacity of rejection of parasitic eggs is found, it would greatly contribute to a better understanding of the coevolutionary process in general and that between brood parasites and their hosts in particular (Sorenson & Payne, 2002).

Modern genetic techniques allow the study of associations between neutral genetic marker [such as microsatellites or single nucleotide polymorphisms (SNPs)] and phenotypes. The basic idea of association studies is that a

marker close to a certain gene influencing on the target trait may also have allele frequency differences between individuals with and without the trait. This is because recombination is less frequent between loci that are close to each other. Therefore, a statistical association between genotypes at a marker locus and the phenotype is usually considered evidence of close physical linkage between the marker and the trait loci (Silverman & Palmer, 2000; Cardon & Bell, 2001; Pritchard & Donnelly, 2001). *A priori*, this methodology may seem too simplistic for the study of complex traits such as behavioural ones, because this approach assumes that genetic differences between individuals with and without the trait would be consequence of differences in expression of one or a few genes. However, recent evidence supports such an approach. Although a vast number of genes could be influencing the expression of a complex trait, only a few loci with large effects (major genes) would be responsible for most of the genetic variation between individuals related to that trait (see Fitzpatrick *et al.*, 2005). There-fore, and by using examples implying behavioural traits, we could distinguish the role of *Gp-9* gene influencing social organization in the fire ant (*Solenopsis invicta*) (Ross & Keller, 1998); or the genetic association between *Pgm* locus and male mating strategy in a marine isopod (*Paracerceis sculpa*) (Shuster & Sassaman, 1997); or those studies showing the influence of the *for* genes on foraging behaviour in several species (reviewed in Fitzpatrick & Sokolowski, 2004).

For purposes other than finding genetic marker of rejection behaviour (i.e. estimation of gene flow among sub-populations), we used a group of polymorphic gen-etic markers (microsatellites) that were tested for genetic association with rejection behaviour by using available specific statistical programs allowing comparison of allelic and genotypic frequencies of nestlings from nests that accepted experimental model eggs and those that rejected them. Therefore, we did not design a specific protocol to detect a genetic association and thus a possible genetic marker of ejection behaviour. However, since we find a significant genetic association with rejection behaviour of

magpies (*Pica pica* Linnaeus, 1758) (see Results) breeding in southern Spain (Guadix) where they are extensively exploited by the brood parasitic great spotted cuckoo (*Clamator glandarius* Linnaeus, 1758) (for a detailed review of this coevolutionary system, see Soler & Soler, 2000), we further test whether differences in rejection rates between different European magpie populations predict differences in allelic frequencies of the hypothe-tical marker, as should be case if the association reflects a true genetic marker (Sorenson & Payne, 2002).

Methods

Study area

Fieldwork was conducted during the breeding seasons 2000–2001 in Hoya de Guadix (37 °18′N, 3 °11′W). The study area is located in Southeastern Spain, at approxi-mately 1000 m a.s.l. and comprises several study plots that vary in size and ecological characteristics (for more detailed information on the Guadix study area, see Soler *et al.*, 1998a,b).

In addition, we also used data and extracted DNA from others European magpie populations sampled for previ-ous studies (Martínez *et al.*, 1999; Soler *et al.*, 1999a). However, mainly due to DNA degradation, some of the samples failed to produce PCR products and, thus, only a few individuals were successfully genotyped for some populations. To avoid spurious results, we arbitrarily used those populations with at least 10 genotyped individuals (i.e. nests). In total, we used DNA of nestlings from six sympatric populations and two allopatric pop-ulations (see Tables 1 and 2). These additional samples were genotyped only for one locus (Ase64, see below).

Field procedures

At the beginning of the breeding season, we systemat-ically explored the study area looking for magpie nests before clutch completion. Once a nest was located, it was visited twice per week and when at least one magpie egg

Table 1 Matrix of genetic distances for Ase64 microsatellite locus (above the diagonal) and matrix of differences in ejection rates (below the diagonal) between populations with 10 or more samples genotyped.

Populations	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	n_{Ase64}	Alleles	Ejection rate (%)
Les Camargues (1)	–	0.077	0.121	0.096	0.108	0.112	0.085	0.137	10	8	18.75
Torres del Segre (2)	19.35	–	0.074	0.034	0.038	0.026	0.065	0.083	14	11	38.10
Logroño (3)	43.75	24.41	–	0.049	0.031	0.056	0.056	0.084	13	8	62.50
Badajoz (4)	25.00	5.66	18.75	–	0.035	0.029	0.052	0.046	22	12	43.75
Guadix (5)	26.25	6.91	17.50	1.25	–	0.031	0.033	0.102	120	12	45.00
Doñana (6)	24.73	5.38	19.02	0.27	1.52	–	0.042	0.075	24	10	43.48
Trondheim (7)*	2.08	21.43	45.83	27.08	28.33	26.81	–	0.109	20	11	16.67
Jyväskylä (8)*	11.61	30.95	55.36	36.61	37.86	36.34	9.52	–	13	7	7.14

Ejection rate, number of analysed individuals and detected alleles per population are shown. Data about ejection behaviour are from Soler *et al.* (1999a).

*Populations in allopatry with the great spotted cuckoo.

Table 2 Matrix of genetic distances estimated from three neutral loci (above the diagonal) and matrix of geographic distances (below the diagonal) between magpie populations with 10 or more samples genotyped.

Populations	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Les Camargues (1)	–	0.101	0.082	0.071	0.073	0.063	0.145	0.156
Torres del Segre (2)	375	–	0.094	0.079	0.099	0.107	0.124	0.144
Logroño (3)	550	225	–	0.046	0.072	0.057	0.154	0.112
Badajoz (4)	1100	700	565	–	0.039	0.038	0.142	0.168
Guadix (5)	950	575	560	385	–	0.050	0.157	0.178
Doñana (6)	1200	800	700	225	300	–	0.193	0.195
Trondheim (7)*	2175	2450	2400	2900	3000	3150	–	0.150
Jyväskylä (8)*	2425	2775	2825	3350	3350	3550	800	–

All values are from Soler *et al.* (1999a).
*Populations in allopatry with the great spotted cuckoo.

was detected in the nest, one experimental model egg was added. Model eggs were made with plaster of Paris and painted resembling those of great spotted cuckoos following the protocol described in (Soler & Soler, 2000). We revisited the nests after 6–7 days and, if the experimental model egg remained in the nest, the pair was classified as an accepter. Otherwise, if the model egg was absent from the nest, the pair was classified as an ejector. To avoid pseudo-replication, nests used in the analyses from 2001 were from magpie territories not controlled in 2000 [magpies are generally faithful to their chosen territory (Birkhead, 1991)]. We only used first breeding attempts because in replacement clutches rejection decisions of egg-recognizer magpies may change to accept experimental eggs due to retaliatory behaviour of the great spotted cuckoo (Soler *et al.*, 1999b). Finally, when nestlings were about 18-day old, we took a blood sample from the brachial vein of magpie nestlings and stored it in 1 mL of 100% ethanol. Afterwards, in order to use only independent samples, we randomly selected one unique nestling sample per magpie nest to perform the genetic analyses.

Laboratory work

Genomic DNA was isolated from blood using the ammonium-acetate precipitation method (adapted from Bruford *et al.*, 1998). We used 11 polymorphic microsatellite loci. Four had previously been isolated from magpies: Ppi1, Ppi2, Ppi3 (Martínez *et al.*, 1999) and Ppi4 (Martinez *et al.*, unpublished data, EMBL accession number: PPI272377); three from Seychelles warbles (*Acrocephalus sechellensis*): Ase12, Ase18 and Ase64 (Richardson *et al.*, 2000); one from indigo bird (*Vidua chalybeata*): Indi28 (Sefc *et al.*, 2001); one from western crowned-warbler (*Phylloscopus occipitalis*): Pocc1 (Bensch *et al.*, 1997); and two from house sparrow (*Passer domesticus*): Pdo5 and Pdo6 (Griffith *et al.*, 1999). Details of the primers used in this study can be found on the Sheffield Molecular Genetics Facility Passerine primer cross-utility database, accessed via http://www.shef.ac.uk/misc/groups/molecol/birdmarkers.html.

Both DNA isolation and genotyping for these 11 microsatellites for samples from Guadix were performed in England (see Acknowledgments) during 2002. Polymerase chain reactions (PCRs) were performed with the forward primer of each marker labelled with a fluorescence dye. The reaction profile for each locus was 94 °C for 120 s, followed by 35 cycles of 94 °C for 30 s, 51–60 °C (depending of each locus) for 30 s, and 72 °C for 30 s; and then 72 °C for 5 min. Volume of PCR reaction were 10 μL, which containing around 10 ng of DNA, 1.0 μM of each primer, 0.2 mM of each dNTP, 1.5 mM MgCl$_2$ and 0.05 units of *Taq* DNA polymerase, in the manufacturer's buffer. The PCR products were electrophoresed through an ABI Prism 377 DNA sequencer (Applied Biosystems). The outputs were analysed using ABI Genescan software (Version 3.1.2) and Genotyper DNA fragment analysis software (Version 2.5).

Genotyping of Ase64 microsatellite locus for magpie chicks from different European magpie populations was performed in France (see Acknowledgments) during 2003. The profile of the PCR reaction was as follow: 94 °C for 120 s, followed by 35 cycles of 94 °C for 15 s, 54 °C for 15 s, and 72 °C for 15 s; and followed by 72 °C for 5 min. Volume of PCR reaction were 10 μL, which containing around 15–50 ng DNA, 0.2 μM of each primer, 0.3 mM of each dNTP, 1.5 mM MgCl$_2$ and 0.25 units of *Taq* DNA polymerase, in the manufacturer's buffer. The forward primer of Ase64 locus was also fluorescently labelled and the PCR products were electrophoresed through an ABI Prims 310 Genetic Analyser (Applied Biosystems). Output was analysed using ABI Genescan software (Version 3.1.2). Ten samples from Guadix with genotypes for Ase64 locus known were genotyped together with these samples from different European magpie populations, thus controlling for possible differences between the two protocols of genotyping.

Statistical analyses

Allelic frequencies (Fisher exact test) and genotypic [log-likelihood (G) based exact test] differentiation analyses

between individual accepters and ejectors from the Guadix population were conduced by using GENEPOP 3.3 software (Raymond & Rousset, 1995). Since deviations from Hardy–Weinberg equilibrium may affect association studies (Pritchard & Rosenberg, 1999; Silverman & Palmer, 2000); we performed Hardy–Weinberg exact tests for each locus for accepter and ejector magpies by using GENEPOP 3.3 (Table 3). Unbiased *P*-values and SE for all above analyses were estimated by using Markov chain with 10 000 dememorizations, 1000 batches and 10 000 iterations per batch. To check whether a certain allele of the Ase64 locus was significantly associated with acceptance or ejection phenotypes, we performed a Pearson χ^2 test with one d.f. considering the number of copies of such an allele in both accepters and ejector pairs against the sum of copies of the remaining alleles.

Since population structure as well as recent population admixture could be a problem for studies of association due to the possibility of character stratification (i.e. different subgroups differing in frequency of traits under investigation; see for instance, Pritchard & Donnelly, 2001; Devlin *et al.*, 2001; Cardon & Palmer, 2003; Zondervan & Cardon, 2004), we tested for stratification of ejection behaviour using STRAT 1.1 software (see also, Pritchard & Rosenberg, 1999; Pritchard *et al.*, 2000) and taking into account all loci except that associated with rejection behaviour. Briefly, this population stratification test performs a χ^2 test for each of the nonassociated microsatellite loci where alleles with fewer than 10 copies were grouped. Differences between allelic frequencies of accepter and ejector individuals were then obtained by summing χ^2 values obtained for each microsatellite locus used. Degrees of freedom were estimated as the addition of those from the individual tests. A nonsignificant result would indicate nonstratification of ejection behaviour in our magpie population.

Finally, to analyse the relationship between matrices of differences in ejection rates of European magpie populations differing in selection pressure from parasitism and matrix of genetic distances between those populations, estimated from the detected genetic marker, we first used GENEPOP 3.3 to generate the allelic frequencies for the Ase64 locus per population. These allelic frequencies were used by GENDIST, included in PHYLIP 3.57c software package (Felsenstein, 1993), providing a matrix of genetic distances (Cavalli-Sforza's chord distance) for Ase64 (Table 1). We also used previously published matrices of geographic distances and genetic distances estimated from three neutral loci for the same magpie populations (Table 2), trying to control for possible geographic or genetic effects. Finally, to explore the relationships between matrices, we used Mantel's and partial Mantel's tests implemented in FSTAT 2.9.3 software (Goudet, 2002), with *P*-values estimated after 2000 randomizations. The matrix of differences in ejection rate was used as dependent variable and all the others as independent variables (for similar analyses see e.g. Soler *et al.*, 2001).

To control for type-I error in multiple tests, we used a modified Bonferroni adjustment. This is because dividing α-value by the number of microsatellites used would be too conservative, because loci are not entirely independent in the genome (see e.g. Silverman & Palmer, 2000; Cardon & Bell, 2001; Freimer & Sabatti, 2004). We then assigned α-value to half of that estimated from Bonferroni correction because probability of detecting type-I error for associated markers was half of that estimated for markers in equilibrium (Cardon & Bell, 2001). Therefore, α-value for our analyses with 11 potential markers was assigned to 0.009. It should be mentioned here that because our genetic samples are from nestlings and not from adults, this level of critical α-value is conservative (see below).

Table 3 Genetic comparisons and Hardy–Weinberg exact tests (HW tests) for each microsatellite locus between accepter and ejector magpies from Guadix.

Locus	Number of alleles	Allelic frequencies	Genotypic frequencies	HW tests	
				Accepters	Ejectors
Ppi1	10	0.03 (<0.001)	0.12 (<0.001)	<0.001* (<0.001)	0.10 (0.001)
Ppi2	19	0.49 (0.002)	0.43 (0.001)	0.91 (0.002)	0.90 (0.002)
Ppi3	8	0.42 (0.001)	0.39 (<0.001)	0.42 (0.002)	0.50 (0.001)
Ppi4	5	0.31 (<0.001)	0.46 (0.001)	<0.001* (<0.001)	<0.001* (<0.001)
Ase12	2	1.00 (<0.001)	1.00 (<0.001)	0.26	0.66
Ase18	12	0.25 (0.001)	0.27 (0.001)	0.21 (0.001)	0.18 (0.002)
Ase64	12	0.002* (<0.001)	0.008* (<0.001)	0.61 (0.003)	0.09 (0.002)
Pocc1	7	0.05 (<0.001)	0.17 (<0.001)	0.001* (<0.001)	<0.001* (<0.001)
Pdo5	7	0.17 (<0.001)	0.18 (<0.001)	0.91 (0.001)	0.77 (0.001)
Pdo6	2	0.41 (<0.001)	0.50 (<0.001)	0.001*	0.07
Indi28	4	0.74 (<0.001)	0.77 (<0.001)	0.06	0.01

Results are *P*-values (SE).
*Significant results after correction for multiple testing.

Results

Allelic frequencies of Ase64 microsatellite (Richardson *et al.*, 2000) significantly differed for magpie nestlings coming from accepter ($n = 55$) and ejector ($n = 45$) nests (Table 3). Further, genotypic frequencies of accepters and ejectors also differed for Ase64 microsatellite locus (Table 3). Those differences were mainly due to two alleles, 429 and 457 bp, the former being more frequent in accepter ($\chi_1^2 = 11.27$, $P < 0.001$, Fig. 1) and the latter in rejecter magpie nests ($\chi_1^2 = 8.56$, $P = 0.003$, Fig. 1).

Those results were not due to stratification of ejection behaviour (see Methods), neither genetically because STRAT software did not detect any stratification ($\chi_{38}^2 = 47.50$, $P = 0.14$), nor geographically because frequency of ejectors and accepters in each sub-zone did not differ from that in the entire Guadix population ($\chi_6^2 = 4.69$, $P = 0.58$).

Moreover, in accordance with the prediction of Ase64 locus being a genetic marker of egg ejection behaviour, we found that genetic distance between populations estimated for this locus explained differences in ejection frequencies among host populations sympatric with cuckoos ($n = 6$, Fig. 2). That was the case even after controlling for genetic distances [from Soler *et al.* (1999a), Table 2] [Partial Mantel's tests, partial correlation coefficient (Ase64 locus) = 0.85, $P < 0.001$, $r^2 = 0.79$] or geographic distances (Table 2) [Partial Mantel's tests, partial correlation coefficient (Ase64 locus) = 0.81, $P = 0.001$, $r^2 = 0.79$]. However, when also including allopatric populations ($n = 2$, Table 1), genetic distances estimated from Ase64 no longer explained differences in ejection rate between populations, neither alone (Mantel's test, $r = 0.35$, $P = 0.08$, $r^2 = 0.12$), even after
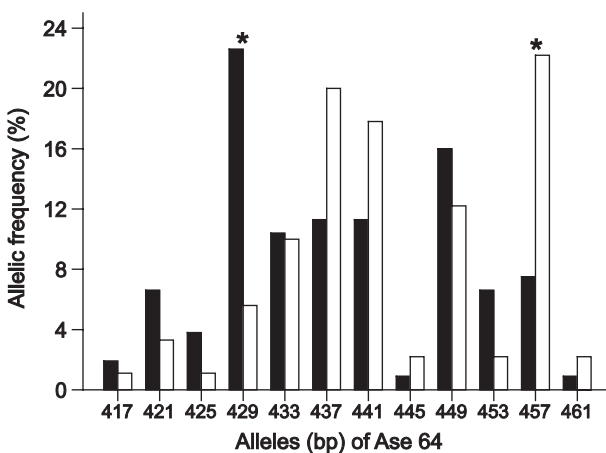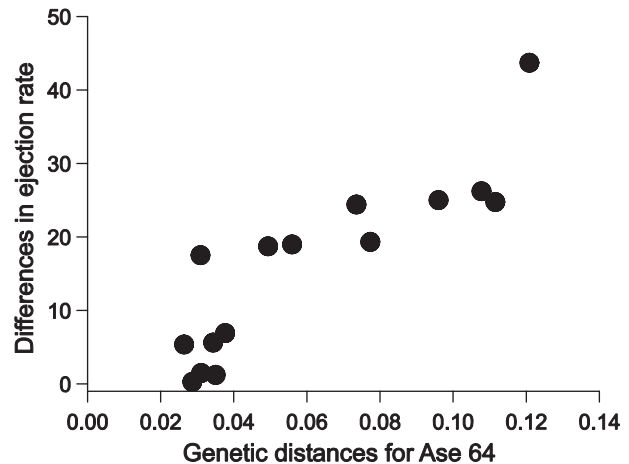

Fig. 2 Relationship between differences in ejection rate (%) and genetic distances for the Ase64 locus for sympatric populations ($n = 15$ differences between six populations) (Mantel's test: $r = 0.88$, $P < 0.001$, $r^2 = 0.78$).

controlling for genetic distance for the three neutral loci in a partial correlation analysis [Partial Mantel's tests, partial correlation coefficient (Ase64 locus) = 0.27, $P = 0.19$, $r^2 = 0.26$], nor when controlling for geographic distance between populations [Partial Mantel's tests, partial correlation coefficient (Ase64 locus) = 0.26, $P = 0.18$, $r^2 = 0.4$].

These results were not biased due to a larger sample size and/or a larger number of alleles detected for the Guadix population compared to that obtained for other magpie populations (see Table 1) because 10 repetitions of the analyses, using randomly selected subsamples from Guadix [with approximately the average sample size used for the other populations being 17 (range of subsamples 12–22, mean 16.9)], gave similar results (Table 4). Therefore, these results strongly suggest an association between Ase64 and rejection behaviour in magpie populations in sympatry with the brood parasite.

Discussion

We found strong statistical association between one (Ase64) of the 11 microsatellite loci analysed and ejection behaviour of mimetic model eggs in magpies. This association was mainly caused by only two alleles, one more frequent in rejecter magpies (457 bp allele, Fig. 1) and another more frequent in accepter magpies (429 bp allele, Fig. 1). The detected association could however appear because of a possible genetic stratification due to gene flow of rejecter magpies from other populations (Soler *et al.*, 1999a). If that were the case, rejecter magpies would be genetically different from accepters due to accepters and rejecters being from different ancestral populations, rather than to any physical association between loci. However, we can discard this possibility because we did not find any

Fig. 1 Allelic frequencies (%) for Ase64 microsatellite locus of nestlings coming from accepter and ejector nests. Symbols refer to alleles whose frequencies significantly differed between accepters and ejectors.

Table 4 Test of the relationship between genetic marker and frequency of ejection using 10 different random samplings from Guadix either, when only sympatric populations (A) or all populations (B) are included in the analyses.

Test	r	P	P_{max}	P_{min}	r^2
A	0.77 (0.03)	0.004* (0.002)	0.018	0.0005	0.59 (0.04)
After controlling for genetic distances	0.74 (0.03)	0.005* (0.002)	0.025	0.0005	0.62 (0.04)
After controlling for geographic distances	0.68 (0.03)	0.01* (0.004)	0.042	0.0005	0.60 (0.04)
B	0.29 (0.01)	0.140 (0.018)	0.257	0.056	0.08 (0.01)
After controlling for genetic distances	0.22 (0.01)	0.257 (0.017)	0.325	0.165	0.23 (0.01)
After controlling for geographic distances	0.22 (0.01)	0.268 (0.020)	0.381	0.171	0.38 (0.01)

Partial Mantel's test was used when statistically controlling for genetic distances from neutral loci or when controlling for geographic distances. All values are averages (SE) except those indicating the maximum (P_{max}) and minimum P-values (P_{min}).
*Significant results.

evidence of genetic stratification in our study population (see Results).

Another alternative explanation is based on geographical stratification of rejection behaviour associated with a relationship between genetic and geographical distances. Our study area is composed of several geographically isolated sub-zones, with different ecological conditions (Soler *et al.*, 1998a), which could influence phenotypic expression of hypothetical egg-recognition genes (e.g. Davies *et al.*, 1996; Brooke *et al.*, 1998). However, rejection rates of particular sub-zones did not differ from that estimated for the entire Guadix population (see Results). Moreover, we found differences in both ejection behaviour and genetic distances for Ase64 locus between sub-zones of Guadix were explained neither by geographic nor by neutral genetic distances. Moreover, we did not find any genetic structure in magpie metapopulation of Guadix, indicating that magpie subpopulations are quite interconnected between them by gene flow (unpublished data). Therefore, a true genetic association between Ase64 locus and gene(s) involved in ejection behaviour of parasitic eggs is the most likely explanation, suggesting physical linkage in the genome.

Moreover, when analysing data from different European magpie populations in sympatry with the great spotted cuckoo, we found a significant positive relationship between population differences in rejection rates and genetic distances estimated for Ase64 microsatellite locus, even after controlling for geographic or genetic distances (from neutral microsatellite loci) (see Results and Fig. 2). However, that was not the case when including data from the two allopatric populations in the analyses. These different results, far from being contrary to the hypothesis of Ase64 microsatellite locus being a genetic marker of ejection behaviour, are what should be expected. Selection pressure due to brood parasitism only occurs in host populations that co-occur with parasites and therefore, the spread of a mutation allowing recognition together with the ancestral genetic environment closest to the mutant gene (i.e. 457 bp allele of Ase64 locus) would only occur in magpie populations subject to brood parasitism, giving rise to the detected association between the 457 bp allele and

ejection behaviour (E′ individuals, Fig. 3). Instead, in magpie populations allopatric with the cuckoo, due to gene flow from sympatric populations (Soler *et al.*, 1999a), there would also be individuals with both the genetic variant influencing on ejection of parasitic eggs and the 457 bp allele of Ase64 (E′ individuals, Fig. 3). However, because no selection pressure favours these ejector mutants in allopatric populations (as far as we know, there is no evidence of intraspecific brood parasitism in Eurasian magpies), relative frequencies of nonejector vs. ejector individuals, all holding the 457 bp allele of Ase64 locus (A′/E′, Fig. 3), should be higher in allopatric than in sympatric population. This larger relative frequency of nonejector individuals holding the 457 bp allele of Ase64 locus (A′ individuals, Fig. 3) in allopatric populations would mask the detected relationship between population differences in ejection rates and genetic distances estimated from the genetic marker for sympatric magpie populations (Fig. 3).

All our results could be considered conservative because we used genetic data from chicks (one per nest) but not from parents, due to logistic problems of
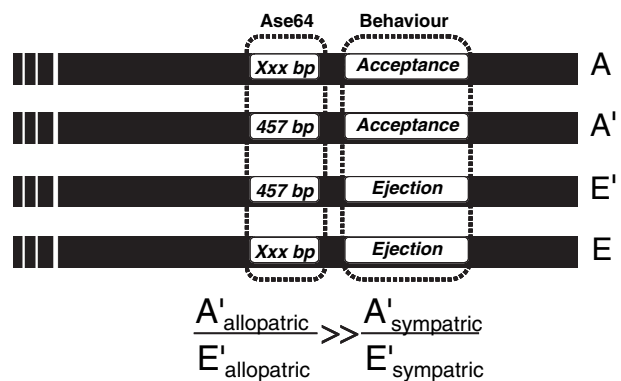

Fig. 3 Genotypes for Ase64 microsatellite locus and the hypothetical gene involved in ejection behaviour. Xxx bp indicates any allele of Ase64 locus except the 457 bp allele. Our results suggest that E′ individuals descended from a mutation in an A′ individual. The difference in proportions of E′ and A′ in sympatric and allopatric populations would be responsible for lack of association when allopatric populations are included in the analysis.

capturing adults, losing information due to segregation of the hypothetical genetic marker of ejection behaviour in the offspring. This is because there is no reason to think that mating preferences would be systematically biased and thus, individual ejectors could be equally mated either with an accepter or with an ejector and *vice versa*. Similarly, phenotypic plasticity due to the associated costs to cuckoo egg recognition and rejection detected for this character (Lotem *et al.*, 1995; Alvarez, 1996; Brooke *et al.*, 1998; Soler *et al.*, 1999a) would partially mask the relationship between genetic marker and phenotypes, making our findings more robust. However, we cannot rule out the possibility that the genetic association detected here includes a genetic basis for a phenotypically plastic decision rule, rather than a genetic basis of such ability to recognize and to reject foreign eggs.

Nothing is known about the genetic mechanism underlying ejection behaviour. It is possible that the genes involved in ejection behaviour in magpies are (or not) the same than those in other species suffering brood parasitism. Therefore, similar studies in other host species could be successful giving crucial information about the genetic control of this behavioural trait. At the other hand and due to this possibility, some caution should be taken when considering the neutrality of Ase64 in previous and future population genetic studies in these host species.

The results presented here should be considered as strong evidence for a genetic influence on egg recognition and ejection, and thus validating a critical assumption of studies on coevolution between hosts and brood parasites. This evidence, however, does not mean that Ase64 was the genetic marker of recognition and ejection of foreign eggs in magpies, which would require further efforts and studies. For instance, the distribution of Ase64 alleles did not completely predict ejection behaviour of magpies and, although it could be a consequence of using genotypes of nestlings rather than those of adults, we cannot rule out the possibility of some other alleles of Ase64 (apart from 429 and 457 bp) and/or even other markers close to other genes acting on expression of this defensive trait apart from that hypothesized to be close to Ase64 locus, were important predictions of the complex behaviour of foreign egg recognition and rejection. In any case, more studies of this locus acting as a genetic marker of ejection behaviour of foreign eggs would be needed to develop a crucial genetic tool in order to test important evolutionary predictions: (i) Tracking fitness consequences of ejector and nonejector host genotypes under different environmental conditions; (ii) studying mechanisms explaining the maintenance of genetic variation in the host and (iii) studying environmental conditions affecting phenotypic expression of gene(s) related to ejection of parasitic eggs, which is a plastic trait (Soler *et al.*, 1999b) that affects the coevolutionary process between brood parasites and their hosts (Davies, 2000).

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