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Can MHC-assortative partner choice promote offspring diversity? A new combination of MHC-dependent behaviours among sexes in a highly successful invasive mammal

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Abstract

Sexual selection involving genetically disassortative mate choice is one of several evolutionary processes that can maintain or enhance population genetic variability. Examples of reproductive systems in which choosers (generally females) select mates depending on their major histocompatibility complex (MHC) genes have been reported for several vertebrate species. Notably, the role of MHC-dependent choice not in mating contexts, but in other kinds of social interactions such as in the establishment of complex social systems, has not yet drawn significant scientific interest and is virtually absent from the literature. We have investigated male and female MHC-dependent choice in an invasive population of North American raccoons (*Procyon lotor*) in Germany. Both male and female raccoons rely on olfaction for individual recognition. Males have an unusually complex social system in which older individuals choose unrelated younger ones to form stable male coalitions that defend territories and a monopoly over females. We have confirmed that females perform MHC-disassortative mate choice and that this behaviour fosters genetic diversity of offspring. We have also observed that males build coalitions by choosing male partners depending on their MHC, but in an assortative manner. This is the first observation of antagonistic MHC-dependent behaviours among sexes. We show that this is the only combination of MHC-dependent partner choice that leads to outbreeding. In the case of introduced raccoons, such behaviours can act together to promote the invasive potential of the species by increasing its adaptive genetic divergence.

Keywords: female mate choice, invasion biology, male coalitions, MHC, *Procyon lotor*

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Introduction

In many animal species, selection has favoured mechanisms driving individuals of one sex not to mate indiscriminately but rather to choose certain partners among potential mates based on phenotypic (or indirectly, genotypic) traits (Trivers 1972). As a general rule, males have a much higher reproductive potential than females, and so competition among the former for access to the latter emerges from this imbalance (Clutton-Brock & Parker 1992). Additionally, females tend to invest more energy in gamete production and parental care than do males (Trivers 1972). Therefore, females are often the 'choosy' sex, taking advantage of male–male competition by preferring to mate with 'better' partners than randomness would provide (Etienne *et al.* 2014). 'Better' can translate into direct benefits for the choosy individuals (Møller & Jennions 2001), but it often means indirect benefits that increase the fitness of the offspring (Trivers 1972; Clutton-Brock & Parker 1992). Typically, females reproduce with males in a

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way that optimizes (Milinski 2003) or maximizes the heterozygosity of offspring (Miller *et al.* 2009; Strandh *et al.* 2012; Winternitz *et al.* 2013; Kamiya *et al.* 2014) with respect to the genes of the major histocompatibility complex (MHC). This is a gene-rich and remarkably polymorphic region in vertebrate genomes with implications not only for the immune response and parasite resistance (Klein 1986), but also for behavioural ecology and population health (Sommer 2005) in all vertebrate groups. An extensive literature now exists reporting cases in which mate choice seems specifically targeted at genes or subregions of the MHC across vertebrate taxa (Milinski 2003; Schwensow *et al.* 2008; Miller *et al.* 2009; Strandh *et al.* 2012; Winternitz *et al.* 2013; Kamiya *et al.* 2014; Roth *et al.* 2014; Santos *et al.* 2016). Preferential mating of MHC-dissimilar pairs is expected to produce offspring with higher fitness (Sommer 2005; Raveh *et al.* 2014). Indeed, in most reported cases of vertebrate MHC-dependent mate choice, females prefer males that are dissimilar or diverse in their MHC (Winternitz *et al.* 2013; Kamiya *et al.* 2014). Nevertheless, examples of assortative female choice (Sin *et al.* 2015) and sex-reversed roles concerning both parental care and MHC-dependent mate choice (Roth *et al.* 2014) provide evidence that disassortative MHC-dependent female choice is not universal.

Given the quality of the MHC as a carrier of genetic information, and since many species are able to detect and interpret MHC-derived cues through olfaction (Yamazaki *et al.* 1979; Wedekind & Füri 1997; Strandh *et al.* 2012), most studies of this topic have implicitly assumed, so far, that whenever individuals of one sex actively chose mating partners, those of the other sex take a passive role. In species with complex social systems, however, in which individuals of both sexes actively participate in choosing members of social groups, we can expect the information content of MHC genes to be used by both males and females. Notwithstanding, to the best of our knowledge, no investigations are available assessing MHC-dependent social choices beyond the canonical mating context.

The North American raccoon (*Procyon lotor*) is a nocturnal carnivore that has long been regarded as asocial and solitary (Barash 1974; Fritzell 1978). Several relatively recent, independent studies have nevertheless revealed a complex sociality (Gehrt *et al.* 2008; Pitt *et al.* 2008; Prange *et al.* 2011; Hauver *et al.* 2013; Hirsch *et al.* 2013a,b; Schuttler *et al.* 2015), with important social interactions particularly among males (Gehrt *et al.* 2008; Prange *et al.* 2011; Hirsch *et al.* 2013a). Raccoon sociality is known to be flexible and to depend on resources, season, kinship, sex and age (Gehrt & Fritzell 1998; Prange *et al.* 2011; Hauver *et al.* 2013; Robert *et al.* 2013). Whereas females tend to be philopatric (Ratnayeke *et al.* 2002; Cullingham *et al.* 2008; Dharmarajan *et al.* 2009) and associate mostly with their own offspring (Ratnayeke *et al.* 2002), males disperse at long distances, interact more with other males, and tend to form male coalitions that generally last for at least a year (Pitt *et al.* 2008; Prange *et al.* 2011; Hirsch *et al.* 2013a,b). Within these small groups (of typically two or three unrelated adults), they may share food and dens (Gehrt *et al.* 2008) and also access to females (Gehrt *et al.* 2008; Hohmann & Bartussek 2011), sometimes over many reproductive seasons (Prange *et al.* 2011). Closely related adult males tend not to associate with each other (Hirsch *et al.* 2013a). Instead of kinship, age seems to play a determinant role in male social interactions (Hauver *et al.* 2013). Typically, older males are dominant, and thus, a social choice might take place when a younger male is exceptionally not rejected but 'accepted' in a coalition. Although males of a coalition defend their territory against other solitary males (Gehrt & Fritzell 1998), they are not able to monopolize breeding with the females in their territories (Hauver *et al.* 2010). In addition to this unusual social system, both males and females of this species have been shown to use olfactory cues for individual recognition (Kent & Tang-Martínez 2014). Raccoons therefore represent an ideal model species for studying MHC-based social choices in both sexes.

Invasive raccoon populations in Germany are thought to derive from a small number of introduction events [between two (Lutz 1995) and four (Fischer *et al.* 2015)] that took place during the last 80 years. They are now relatively common in central Europe (Lutz 1995; Beltrán-Beck *et al.* 2011; Fischer *et al.* 2015). In addition to ecological aspects, invasive species are undoubtedly appealing for research from an evolutionary perspective, because of the genetic and behavioural processes that allow some species to thrive and become invasive despite the generally low number of founders (Frankham 2004; Roman & Darling 2007). As the latter is associated with genetic bottlenecks, strong drift, low genetic diversity and reduced fitness (Tsutsui *et al.* 2000), a theoretical conflict emerges that has been referred to as the genetic paradox of introduced species (Frankham 2004; Roman & Darling 2007). Increased propagule pressure due to multiple introduction events has been shown to facilitate invasion success (Simberloff 2009), but empirical reports on other 'solutions' to this paradox are surprisingly rare in the literature.

Here, we have tested the hypothesis that mate choice and social choices can act together and lead to more genetic diversity. A social system that is particularly efficient in terms of outbreeding might facilitate the invasive success of a species. Therefore, we have investigated the role of the MHC class II allelic repertoire in

mate choice and social choices and whether these choices have promoted adaptive genetic diversity in offspring in a sample of 146 raccoons live-trapped in northeastern Germany.

We have detected an MHC-dependent interaction pattern, both among males in coalitions and between sexes in mating couples, but with individuals of the two sexes choosing in opposite manners. This is the first time, as far as we are aware, that MHC-dependent choice has been described as occurring simultaneously among males and females. The observed pattern fits a behavioural combination expected to lead to outbreeding. Our results imply that ecological success, including invasive potential, can be related to the synergic MHC-dependent choice behaviours of males and females. We could additionally identify the probable North American geographic origin of this introduced German raccoon population.

Materials and methods

Study area, sampling and radio tracking

All individuals ($N = 146$) were captured in the Müritz National Park area in northeastern Germany (53°15′–22′ N and 13°8′–21′ E) over the course of 6 years (2006–2011). Live-trapping took place at 53 sites corresponding to 4.8 sites per 100 ha in a study area of 1114 ha, as described elsewhere (Michler *et al.* 2015). Upon trapping, all individuals were ear-tagged, weighed, aged, sexed and sampled. In the absence of predators, invasive raccoons in central Europe can live for more than 10 years. The age of all animals was estimated based on two types of dental analysis: dental abrasion for adults (Grau *et al.* 1970) and tooth eruption for juveniles (Montgomery 1964). The reference for the age estimation was built by 40 marked raccoons that were aged postmortem using dental analysis as well as the ossification of skull joints (sutures). The ageing is thus expectedly highly reliable, with an estimated error range of 1 year (95% confidence). Tissue samples were collected for DNA extraction (*microRheologics* kits) through buccal swabs and one skin punch of ca. 6 mm in diameter from each animal, the skin samples being kept in 99.8% ethanol at 4 °C until extraction. Individual movement patterns and the territorial and social behaviours of males and females were monitored by terrestrial very high-frequency (VHF) radio telemetry (149 103–151 042 MHz) with radiocollars from Biotrack (Dorset, England) and Wagener (Cologne, Germany). We radiocollared 69 individuals (36 males and 33 females) and analysed 31 202 individual locations during the whole sampling period (Michler 2016). Locations were determined using antennas that were hand-held or mounted on one of seven automobile or three boat units. Fifteen camera traps placed across the park yielded additional location data (5365 phototrapping nights and 18 721 images of 183 individuals). Static and dynamic interaction analyses using Jacobs' index (Jacobs 1974) were employed to determine social organization, such as raccoon pairs and coalitions. The raccoon density in the area ranged from four to six individuals per 100 ha (summer measurements, including offspring; Michler 2016). All animal handling complied with current German law (§5, Tierschutzgesetz).

MHC class II genotyping by next-generation sequencing

We amplified 188 base pairs of the exon 2 of MHC class II DRB loci of each individual, in replicates, using the primers CS1-DRB-5c (TCGCCGCTGCACCGTGAAGCT) and CS2-DM2 (GCACCCGCTCCGTCCCATTGA). Two technical replicates per individual were processed independently. The polymerase chain reactions (PCRs) were set at a volume of 25 μL, with 2.5 μL PCR buffer containing $MgCl_2$, 1 μL each primer (10 μM), 1 × FastStart High Fidelity Taq DNA polymerase (Sigma-Aldrich), 0.2 mM each dNTP, 2.5 μL GC-Rich and 14.9 μL H_2O, under the following conditions: initial 7 min denaturation at 95 °C and 30 cycles of denaturation (1 min at 95 °C), annealing (1 min at 55 °C) and extension (1 min at 72 °C). The second PCR for index incorporation was set at a volume of 20 μL (2 μL PCR buffer, 3.6 μL $MgCl_2$, 1 μL DMSO, 1 μL each primer (10 μM), 1 × FastStart High Fidelity Taq DNA polymerase (Sigma-Aldrich), 0.2 mM each dNTP, 4 μL barcode mix, 1 μL PCR product, and 6.6 μL H_2O) under the following conditions: initial 10 min denaturation at 95 °C and 10 cycles of denaturation (15 s at 95 °C), annealing (30 s at 55 °C) and extension (1 min at 72 °C). Indexing was performed with a Fluidigm Access Array Barcode Library for Illumina Sequencers-384. Isomolarity for the downstream steps was achieved by measuring the DNA concentration with a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) and adjusting volumes accordingly with H_2O. Purification was performed with the MinElute 96 UF PCR Purification Kit (Qiagen). Sequencing was performed in a MiSeq instrument (Illumina, Inc.).

We followed our previously published bioinformatics pipeline (Sommer *et al.* 2013), with a few modifications to accommodate Illumina reads. Results were checked for artefacts by first removing low-quality reads (mean Phred quality score <20) and reads with any sequencing errors on primers or barcodes. All reads were then submitted to local BLAST against MHC class II sequences, and clustering was performed in QIIME (Caporaso *et al.* 2010) with default settings. Putative chimeras were

detected and removed using UCHIME (Edgar *et al.* 2011). The MHC allele calling of each sample was based only on variants that passed our conservative approach and had been identified in both replicates (originated in independent PCRs). To assess reproducibility, the whole bioinformatics pipeline (from raw data to allele calling) was also independently performed with a stepwise threshold clustering (STC) approach (Stutz & Bolnick 2014).

Allele phylogeny

We inferred the phylogenetic relationships between all allele amino acid sequences found here using a Bayesian approach implemented in MRBAYES 3.2.6 (Ronquist *et al.* 2012), with a Poisson amino acid model and burn-in chain length of 1.1×10^6.

Relatedness and parentage

In a previous study (Gramlich *et al.* 2011), 10 microsatellite loci had already been genotyped for 141 raccoons (74 males, 67 females) of the present sample. The current data set was completed by genotyping five further individuals. Detail on the characterization of these markers was published before (Cullingham *et al.* 2006; Fike *et al.* 2007) and is provided in the online supplemental material. Genotyping failed for one locus in one individual and for three loci in another individual. Both these individuals were exclusively offspring in our analyses, meaning that they were not involved in any mother–father pair nor in any male coalition. All other individuals were successfully genotyped for all 10 microsatellites. Based on these genotypes, we estimated pairwise relatedness indices between all possible pairs of individuals using ML-RELATE (Kalinowski *et al.* 2006).

We were able to define 95 mother–father–offspring trios using CERVUS 3.0.3 (Marshall *et al.* 1998) (average confidence of 94.92% for mothers and 91.43% for fathers, SD = 5.85 and 6.49, respectively). We run CERVUS with 10^5 simulations, allowing for 10% unsampled parents, and considered all ten microsatellites, from which individuals had to be genotyped in at least nine. We allowed for 1% genotyping error rate. The number of candidate fathers in all paternity tests ranged from 2 to 29 (mean = 23.21, SD = 7.18). We repeated the parentage analyses with COLONY2 (Jones & Wang 2010) reaching 97.9% agreement between methods. Parentage is particularly robust in our sample because of field observations (supporting the assignment of 86 maternities) and because of male long-distance dispersal. As a consequence, relatedness within groups of assessed males for paternity was relatively low. For instance, there were no full-siblings of offspring among its candidate fathers, and no two full brothers were candidate fathers of the same offspring. After filtering out the individuals not genotyped for the MHC, the trios consisted of 95 unique offspring, 20 unique mothers and 17 unique fathers.

Female mate choice and male choice for coalition partners

The parentage information was used as basis for the partner choice analyses. We assumed each offspring to be the outcome of an actual female choice event. To account for litter sizes, we considered the identity of mothers and fathers as random effects in all statistical modelling (see Statistics below). In 64 cases out of the 95 established family trios (see Relatedness above), at least one further sexually mature male was known to be resident in the study area during the time of conception (63 days $\pm$ 3 days before birth). These males were thus 'available' (in space and time) for each female and they were therefore considered as potential mates for each female. By combining this data set with the MHC class II genotyping data, we were able to investigate 60 MHC-genotyped groups, each of which consisted of one family trio and at least one further candidate male. Each group had an average of 19.22 potential fathers in addition to the real (i.e. biological) father (ranging from 15 to 22, SD = 2.86). Thus, the final female choice data set amounted, in total, to 60 real choices (mother and biological father) and 1213 potential choices (mother and potential mates).

Additionally, telemetric observations enabled us to establish eight male coalitions. These were defined as extremely close, stable and long-term interactions (more than 75% overlap of action space in 1 year) between two or more adult males (Gehrt & Fritzell 1998; Gehrt *et al.* 2008; Pitt *et al.* 2008; Hohmann & Bartussek 2011). Based on the literature (Hauver *et al.* 2010) and on own observations, we considered the older male of each coalition as being dominant (chooser) and the young male(s) as the chosen one(s). All other solitary males known to live in the same area by the time of establishment of each coalition were considered 'potential' choices for each older male in a coalition. All but one coalition (three males) consisted of two males. The number of potential males for each coalition ranged from 15 to 27 (mean = 21.75, SD = 4.46). This constituted the data set used for male choice analyses (nine real choices and 174 potential choices). In 29 cases out of the 95 established trios, the father belonged to a male coalition. Multiple paternities were observed in ten litters: in eight cases, two males sired the offspring, and three males sired the offspring in the remaining two cases. In all cases of multiple paternities, the fathers belonged to the same male coalition.

Defining MHC dissimilarity and diversity parameters

We aimed at testing whether female or male raccoons chose their mating or coalition partners depending on their MHC constitution. The allelic diversity and amino acid distance among individual MHC alleles, especially in the functionally important antigen-binding sites, was used as a surrogate for the range of pathogenic antigens that could be presented to T cells (Sommer 2005). We calculated four MHC dissimilarity parameters for each pairwise comparison (which were female–male pairs concerning mate choice and male–male pairs concerning coalition partner choice) and two MHC diversity parameters for every male that was a real or potential father or coalition partner. Whereas the dissimilarity indices implied a comparison between the alleles of the assessing and the assessed individuals, the diversity indices were inherent to each assessed individual. In the female choice analysis, the candidate males were the assessed individuals, whereas the females were the assessing individuals. Conversely, in the male coalition partner choice analysis, younger males were assessed by older males, which were the assessing individuals. The six MHC dissimilarity or diversity parameters were defined as follows (Santos *et al.* 2016):

1 Male Allele Dissimilarity (MALDis): number of MHC alleles present in the assessed individual but not shared by the assessing individual.
2 Couple Allele Dissimilarity (CALDis): sum of the number of nonshared MHC alleles in each pairwise raccoon comparison.
3 Mean Amino Acid Dissimilarity (μAADis): mean amino acid distance (defined as the number of amino acid substitutions per site using the Poisson correction model) among the nonshared MHC alleles in each raccoon pairwise comparison.
4 Antigen-Binding Site Dissimilarity (ABSDis): the same as μAADis, but considering only the 21 putative antigen-binding site residues, as defined elsewhere (Castillo *et al.* 2010).
5 Male Allele Diversity (MALDiv): number of different MHC alleles present in each male.
6 Male Amino Acid Diversity (MAADiv): sum of amino acid distances among the MHC alleles of each male.

Defining microsatellite dissimilarity and diversity parameters

We also investigated the genetic dissimilarity among pairs of individuals and the genetic diversity of single adults and offspring in terms of evolutionarily neutral markers. For these aspects, we calculated the following indices based on 10 unlinked microsatellites (Gramlich *et al.* 2011):

1 Pair Microsatellite dissimilarity (PMsDis): proportion of nonshared microsatellite alleles in each raccoon pairwise comparison, in relation to the total number of alleles in the pair.
2 Microsatellite diversity (MsDiv): proportion of heterozygous microsatellite loci in relation to the total number of microsatellite loci genotyped, in each individual.

Statistics

Following a two-step method recently established by us (Santos *et al.* 2016), we employed two independent statistical approaches to test the hypothesis of MHC-dependent mate or social choice among females or males:

1 Monte Carlo randomization tests: we first asked whether females or males chose partners in a way that differed significantly from randomness by comparing the observed means of each MHC index to randomized means obtained by Monte Carlo simulations (Sin *et al.* 2015). For each offspring, we calculated the mean of the MHC genetic indices for all real pairs. We then created a distribution for each MHC genetic index by disassembling all pairs and reassembling them randomly 10^4 times (using, for each offspring, the set of candidate males available to the mother or, in the case of male coalitions, the set of younger males available to the older male). *P*-values were calculated as the proportion of times in which the simulated means were further away from the mean of their distribution than was the observed mean, in either direction (two-sided tests). The resampling method was also used to test whether the observed genetic relatedness within coalitions and the observed offspring microsatellite diversity differed significantly from that expected by chance.
2 Generalized linear mixed models (GLMMs): independently, we asked whether the likelihood of any male being chosen by a female (in the cases of female mate choice) or by an older male (in the cases of male choice for a coalition partner) could be predicted by any of the MHC indices. For each offspring, we calculated all six MHC genetic parameters corresponding to its real parents (real female choice), each of its potential parents (potential female choices), the real coalition partners (real male choice), and the potential coalition partners (potential male choices). The calculated results of the MHC genetic parameters were organized into one row per choice, and the value of a

binary variable called 'CHOICE' was set to '0' or '1', depending on the pair being potential or real, respectively. GLMMs were used here because the same females and the same males were involved in several 'choice' events, which were not necessarily independent from each other. Female and male identification codes (as well as sampling year) were therefore included as random factors in the models. In R syntax, our models were built using the *glmer* function of the LME4 package (Bates *et al.* 2015) with the following formula:

```
glmer(CHOICE~MHC.INDEX+(1|ASSESSED.ID)+
(1|ASSESSING.ID)+  (1|SAMPLING.YEAR),family=
"binomial")
```

Notably, the resampling method is fundamentally different from the GLMM approach in the sense that it is 'blind' to the degree of dissimilarity or diversity between females and males (or between two males) but, instead, takes into account the binary categories 'more' or 'less' dissimilar/diverse than another given random male would be.

Finally, as multiple paternity was observed in ten litters, we tested (with generalized linear models and the Poisson distribution) whether breeding success (measured by the number of offspring) among the two or three coalition males depended on (i) any of the four MHC dissimilarity parameters with respect to the parents in each litter, on (ii) any of the two MHC diversity parameters of males, or on (iii) the age of the males.

Results

Quality control and MHC genotyping

Sequencing yielded 31 704 421 reads. After quality filters and merging of forward and reverse reads, the merged sequences were clustered to 35 sequence variants which were assigned to 143 individuals (98% of 146 sampled raccoons). The application of a chimera filter (Edgar *et al.* 2011) led to the exclusion of four variants. Removal of singletons led to the exclusion of nine further variants. The remaining 22 sequence clusters were considered real MHC alleles. The reproducibility between our pipeline (Sommer *et al.* 2013) and the STC (Stutz & Bolnick 2014) was 95.55%. We found between three and six MHC alleles per individual (average = 4.08, SD = 0.97), indicating a minimum of three loci. No stop codons and no shifted or disrupted reading frames were observed. The final MHC genotyping data of all individuals investigated here are available as an electronic table at the Dryad Digital Repository.

Geography and phylogeny

Twelve (54.55%) of the 22 alleles found in our sample had previously been described in North American raccoons from the Alabama/Georgia region (Castillo *et al.* 2010) (*Prlo-DRB**01, *DRB**02, *DRB**10, *DRB**14, *DRB**16, *DRB**19, *DRB**25, *DRB**28, *DRB**62, *DRB**69, *DRB**71 and *DRB**102, with the GenBank accession nos. GU388312, GU388313, GU388321, GU388325, GU388327, GU388329, GU388334, GU388337, GU388354, GU388356, GU388358 and GU388368, respectively). Some of these alleles had also been observed in other North American areas (Castillo *et al.* 2010). The remaining ten alleles were assigned new designations, from *Prlo-DRB**254 to *Prlo-DRB**263, ordered sequentially according to abundance in our sample. An overview of the sequences and abundances of the 22 MHC alleles found here is given in Table S1 (Supporting information). The phylogenetic relationships between them are depicted in Fig. 1.

Female mate choice

Our study provided clear evidence for MHC-dependent disassortative female choice increasing the functionally important amino acid diversity in the MHC repertoire of the offspring. The Monte Carlo randomization tests indicated that female raccoons chose males that were more dissimilar from themselves than a random male would be concerning one amino acid-based parameter (ABSDis, *P*-value = 0.0009), with a tendency for μAADis (*P*-value = 0.0821) (Fig. 2). Further, females mated with males that were more diverse than expected by chance, also concerning the amino acid-centric parameter (MAADiv, *P*-value = 0.0277). No evidence for a deviation from randomness was observed when considering the genotype-based parameters (MALDis, CALDis, or MALDiv, all *P*-values >0.8; Fig. 2).

The GLMMs confirmed the results obtained by the randomization analyses. They also indicated a positive association between the probability of being chosen and dissimilarity/diversity measured by the amino acid-based parameters ABSDis and MAADiv (*P*-values = 0.0019 and 0.0188, respectively) and a tendency for μAADis (*P*-value = 0.0855). No deviation from randomness could be inferred for the remaining indices (MALDis, CALDis and MALDiv, with *P*-values = 0.9799, 0.7710 and 0.9370, respectively). Detailed results for these tests are given in Table S2 (Supporting information).

Effect of MHC-dependent female choice on offspring genetic diversity

We used the randomization approach to test whether the genetic diversity among offspring (MsDiv) was

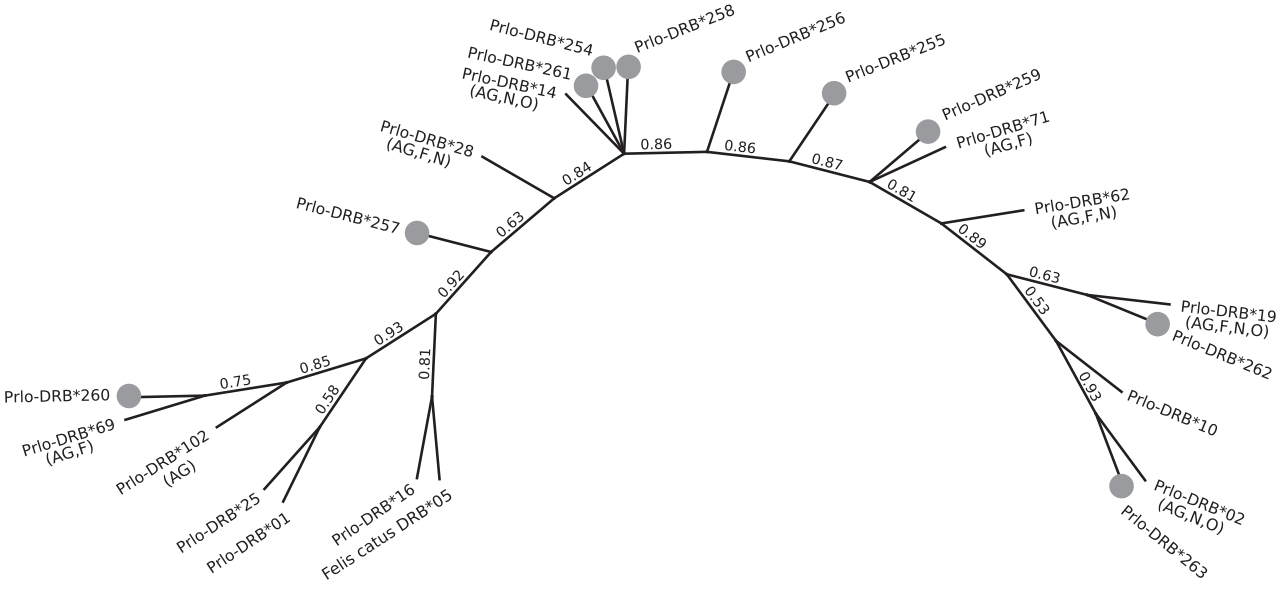

Fig. 1 Phylogenetic relationship of the 22 MHC class II amino acid alleles observed in German raccoons. The 10 *Prlo-DRB* alleles found so far only in Germany are indicated by a grey circle at the corresponding branch tips. Alleles that have also been found previously in North American raccoons (Castillo *et al*. 2010) are labelled according to the geographic region of detection (AG: Alabama/Georgia; F: Florida; N: New York; O: Ontario) if they are phylogenetically close to alleles private to Germany. The DRB*05 allele sequence of the domestic cat (GenBank accession no. AAB65572) was used as outgroup. Bayesian posterior probabilities are provided for every internal branch.

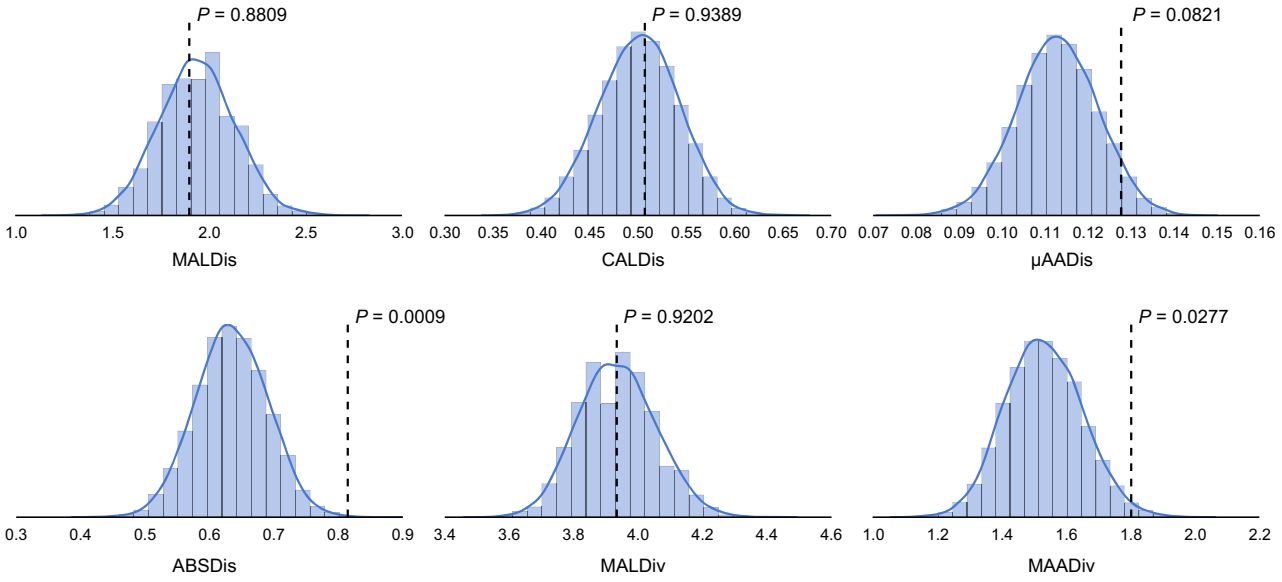

Fig. 2 MHC-dependent female choice tested with Monte Carlo simulations. The frequency distributions of the means of the six MHC parameters calculated from 104 Monte Carlo randomizations of potential female–male couples are given as blue bars. The mean values for the real breeding couples (MALDis, CALDis, μAADis and ABSDis) or real fathers (MALDiv and MAADiv) are given in each plot as a dashed vertical line, and the corresponding *P*-values are indicated.

influenced by the MHC-dependent mating behaviour of the females. The actual observed microsatellite diversity was significantly higher than expected if females chose their mates randomly (*P*-value = 0.0223; Fig. 3).

Male choice for coalition partners

Our study provided clear evidence for MHC-dependent assortative male choice for coalition partners. Monte

Carlo randomization tests indicated that males built a coalition with partners that were more similar to themselves than expected by chance, with respect to all four MHC dissimilarity parameters (for MALDis, CALDis, μAADis and ABSDis, *P*-values = 0.0492, 0.0242, 0.0266 and 0.0437, respectively) (Fig. 4). Accordingly, they preferred younger males with low amino acid distances among the MHC alleles (MAADiv, *P*-value = 0.0006).

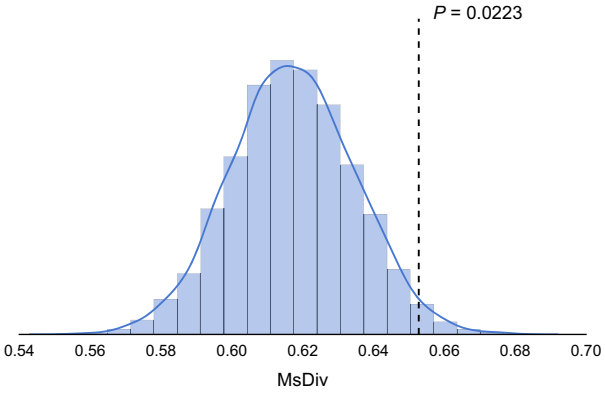

Fig. 3 Effect of female choice on genetic diversity of offspring. The mean microsatellite diversity (MsDiv) of the observed offspring is given as the dashed vertical line. The frequency distribution of the mean MsDiv of simulated offspring (generated by random mating of females with potential mates), calculated from 10^4 Monte Carlo randomizations, is given as blue bars. The corresponding *P*-value is indicated.

Thereby, the number of different MHC alleles present in each male did not play a role in the selection process (MALDiv, *P*-value = 0.8999).

Again, GLMMs confirmed the results obtained by the randomization analyses. Statistical modelling indicated a significant correlation between the same five MHC indices (MALDis, CALDis, μAADis, ABSDis and MAADiv) and the probability of a male being chosen (also, here, in the direction of similarity and less diversity, with *P*-values = 0.0240, 0.0091, 0.0170, 0.0166 and 0.0137, respectively). Once again, no evidence for an effect of MALDiv was detected (*P*-value = 0.1160). Detailed results for these tests are given in Table S3 (Supporting information).

Using randomizations with respect to neutral microsatellite alleles, we then tested whether the individual relatedness within coalitions was different from expected by chance. We found that relatedness among males in real coalitions was significantly lower than that expected under random male choice for coalition partners (*P* = 0.0316; Fig. 5). Further details on the quality of MHC and microsatellite markers assessed here are given in Tables S4 and S5 (Supporting information).

Effect of age and MHC on reproductive success within coalitions

We did not find any statistical support for a relationship between the age of males and the number of offspring that they produced in litters with multiple

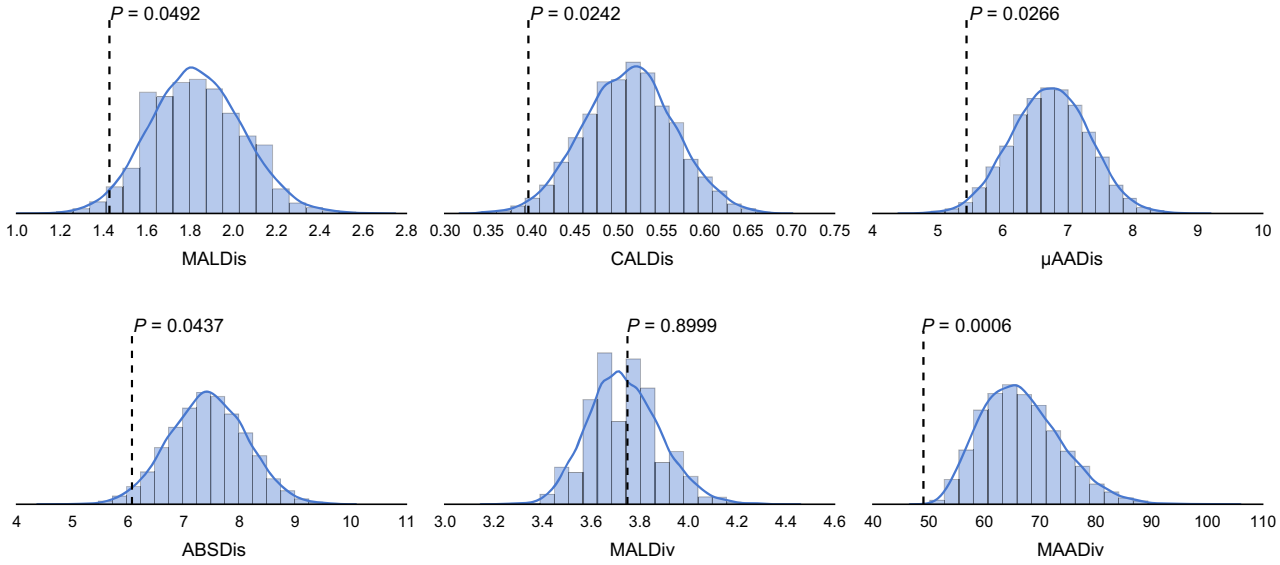

Fig. 4 MHC-dependent male choice for coalition partners tested with Monte Carlo simulations. The frequency distributions of the means of the six MHC parameters calculated from 10^4 Monte Carlo randomizations of potential male–male pairs are given as blue bars. The mean values for the real coalition pairs (MALDis, CALDis, μAADis and ABSDis) or chosen coalition partners (MALDiv and MAADiv) are given in each plot as a dashed vertical line, and the corresponding *P*-values are indicated.

paternity (maximum-likelihood test, *P*-value = 0.55). In other words, older and younger males within a coalition did not differ in their reproductive success. Similar results were found concerning the effect of individual male MHC constitution on the number of offspring per litter (maximum-likelihood tests, *P*-values = 0.23, 0.23, 0.87, 0.88, 0.99, 0.99 and 0.55, for MALDis, CALDis, μAADis, ABSDis, MALDiv and MAADiv, respectively).

Discussion

We describe here a system in which MHC class II genes simultaneously affect social choices of males (when choosing a male coalition partner) and mate choice of females, but with individuals of the two sexes choosing in opposite manners. Whereas females choose dissimilar and diverse mates, males choose partners in an assortative manner. We have been able to show mathematically (using randomizations) that the MHC-disassortative mating pattern created by the combination of male and female behaviour leads to genomewide genetic diversity among the offspring. Additionally, we have determined the southwest of the United States as the most likely geographic origin of this German raccoon population by identifying common and related MHC class II alleles.

MHC-dependent female mate choice and male partner choice are antagonistic but not conflictual

We found evidence for MHC-disassortative female choice, as females reproduced more often with males that carried more dissimilar MHC alleles at the amino acid level. Females also preferred males carrying more diverse MHC alleles, that is males with a higher intra-individual amino acid diversity than randomly assigned males. The preference was even more evident when the analyses were restricted to the 21 positively selected antigen-binding residues (ABSDis; Fig. 2) involved in antigen recognition and presentation. A likely outcome of MHC-dependent female choice is a higher functionally important amino acid diversity in the MHC repertoire of the offspring. The kind of disassortative female choice for mates observed here is, in general terms, in agreement with several previous studies that have assessed MHC-dependent mate choice in the laboratory and under natural conditions in wildlife (Schwensow *et al.* 2008; Miller *et al.* 2009; Strandh *et al.* 2012).

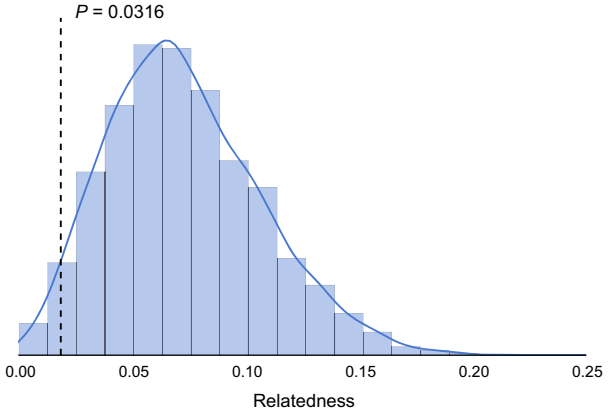

Fig. 5 Effect of male choice on genetic relatedness within male coalitions. The mean relatedness within the observed coalitions is given as a dashed vertical line, whereas the blue bars represent the frequency distribution of the mean relatedness from simulated male coalitions, calculated from 10^4 Monte Carlo randomizations. The corresponding *P*-value is indicated. Relatedness indices were calculated with ML-Relate (Kalinowski *et al.* 2006).

We have also found that males build coalitions depending on their MHC class II alleles. Among raccoons, both males and females have been recently shown to discriminate individuals through smell (Kent & Tang-Martínez 2014). Males and females probably use a common substrate for olfactory choice, such as urineborne MHC molecule fragments or MHC-dependent bacteria. Thus, from a physiological point of view, it is not surprising that males are also able to choose their coalition partners according their MHC alleles. However, unlike females, older males perform assortative male choice for a coalition partner, as older males accept younger coalition partners that are less different from them (and also less diverse) than from randomly assigned males (Fig. 4). As with females, the two different statistical approaches employed here (randomizations and modelling) have converged to concordant results. Interestingly, although males were found to choose more similar males than expected, this did not correlate with choices for more relatedness. Indeed, as depicted in Fig. 5, males seemed to have avoided direct relatives. This is in agreement with previous work that observed raccoons avoiding kin as coalition partners (Gehrt *et al.* 2008; Hirsch *et al.* 2013a). As a consequence, male coalitions tend to be simultaneously more homogeneous than expected by chance concerning the adaptive MHC genes (Fig. 4) but more heterogeneous than expected by chance concerning the rest of the genome (assessed through microsatellites; Fig. 5).

Using the mating outcome (offspring) of naturally paired individuals as a proxy for mate choice, we cannot completely rule out that our estimates of female mate choice were biased by the occurrence of postcopulatory mechanisms, such as selective abortion, sperm competition or cryptic female choice.

In evolutionary terms, shared access to females and shared paternities are a fairly high cost paid by adult males when accepting an unrelated coalition partner.

Thus, the benefits of being in a coalition are expected to offset such costs. The evolutionary meaning of male coalitions has been often discussed (Gehrt *et al.* 2008; Prange *et al.* 2011; Hauver *et al.* 2013), and the proposed benefits of this social form converge to coalitions being better at defending territories (and reproduction with the females therein). As male dispersal takes place early in life (typically in the first year), males are unlikely to meet close male relatives in adulthood (Gehrt *et al.* 2008). Thus, under the pressure of building a coalition and in the absence of direct relatives, older males seem to choose younger males that resemble themselves the most (by the sense of smell, which reflects MHC alleles). In our sample, the average measured male natal dispersal distance was 42 km (ranging from 3 to 285 km, $N = 38$).

The predicted effect caused by the four possible combinations of MHC-dependent disassortative and assortative behaviours of males (regarding coalition partners) and females (regarding sexual partners) on offspring genetic diversity is given in Table 1. The only combination leading to outbreeding involves males performing MHC-assortative choices for coalition partners and females performing disassortative choices for mates (or male coalitions). Thus, although, at first glance, the antagonistic partner-choosing behaviour of males and females might suggest a type of sexual conflict, the opposite is likely to be taking place. The increased MHC homogeneity of males in a coalition harmonizes with the female behaviour of disassortatively choosing a male or a coalition. In this sense, the MHC-dependent coalition partner behaviour of the males can enrich the outcome of the MHC-dependent female choice behaviour by increasing its genomic outbreeding efficiency. No sexual conflict, but an alignment of interests from both sexes leading to more variability of the litter, seems to be the outcome among raccoons. This combination of disassortative and assortative behaviours thus renders an evolutionarily stable mechanism.

We have tested whether outbreeding is detectable regarding the microsatellites and have confirmed this expectation: the offspring sampled here are more diverse than they would have been without biased mate choice (Fig. 3). This is, as far as we know, the first time that the effects of MHC-disassortative mate choice have been demonstrated on the overall genetic diversity of offspring based on simulation tests. It illustrates the outbreeding potential of this mating behaviour among raccoons and leads to the conjecture that it probably helped raccoons overcome the immediate inbreeding constraints at the beginning of the expansion of this species in Europe.

Despite the costs of outbreeding in terms of local adaptations, outbreeding is likely to be more evolutionarily stable than inbreeding, especially in species that go through strong density fluctuations. Outbreeding can be particularly valuable for species that are regularly confronted with genetic bottlenecks. Raccoon populations in the United States have been reported to go through cyclical rabies virus outbreaks, on average every fourth year, which can wipe out up to 85% of the population (Biek *et al.* 2007). Thus, part of the success of this and other thriving German raccoon populations, despite the initial low number of individuals (Michler & Michler 2012; Frantz *et al.* 2013; Fischer *et al.* 2015), is possibly a side effect of the adaptive history of this species in North America. Future work should reveal whether the same behavioural pattern is observed, as expected, in outbred American raccoon populations or, even more interesting, in other, independently introduced populations of raccoons such as those in Japan (Ikeda *et al.* 2004), Belarus and Azerbaijan (Geptner *et al.* 1988). This high MHC diversity despite cyclic population bottlenecks has been identified previously (Winternitz *et al.* 2014; Schuster *et al.* 2016), but the authors have failed to demonstrate a mechanism leading to diversity. Our work provides one.

Table 1 Layout of the four combinations of male and female choosing behaviour for coalition partners and mates, respectively, with their corresponding outcome with respect to offspring diversity

♀ choice	♂ choice	Outbreeding
≠	≠	✗
≠	=	✓
=	≠	✗
=	=	✗

=: assortative partner choice; ≠: disassortative partner choice; ✗: no; ✓: yes. Only one combination (shaded line) leads to outbreeding.

The geographic origin of raccoons is most likely Alabama/Georgia

Major histocompatibility complex allele composition, their frequencies and their phylogenetic relationships can shed light on the ancestry and migration history of species (Vina *et al.* 2012). Out of the five North American regions with detailed available raccoon MHC class II genotype data [Alabama/Georgia, Florida, Missouri, New York and Ontario (Castillo *et al.* 2010; Ruiz-López *et al.* 2014)], the population in Alabama/Georgia is the only one harbouring all 12 North American alleles found in our sample. Our phylogenic analysis has revealed that the alleles that have only been observed in Germany (*Prlo-DRB**254 to *263), so far, are closely related to the North American alleles *Prlo-DRB**02, *14,

*19, *69 and *71, and again only the Alabama/Georgian population harbours all these five alleles. All other populations lack at least two of them. Additionally, the allele *Prlo-DRB**102 has been, until detected in our sample, private to the Alabama/Georgia population. Thus, the source population of the raccoons that inhabit northeastern Germany today most likely descended from the region comprising the Alabama and Georgia states. Although the number of raccoon introductions in Germany has been recently corrected to at least four (Fischer *et al.* 2015), the population assessed here shows no evidence of genetic structure (Balkenhol *et al.* 2011) or admixture with other German raccoon populations (Fischer *et al.* 2015). Instead, it appears as a discrete cluster in previous population structure analyses (Frantz *et al.* 2013; Fischer *et al.* 2015), supporting the hypothesis of a single introduction event in the 1940s (Frantz *et al.* 2013).

Final remarks and outlook

Although invasive species are a common subject in ecological literature, we still do not understand what makes some species more able than others to become invasive. The increased propagule pressure associated with multiple invading individuals or multiple invasion events seems to be currently the best accepted theoretical framework to explain the 'invasive potential' of a species. In this work, we have presented evidence for a system that promotes MHC and genomic diversity by combining female and male social behaviour in a more complex manner than previously described. By enhancing the generation of genetic variability, the synergic behaviour of males and females can also facilitate the rapid (re)colonization of territories. An interesting prediction that follows from this statement is that species that engage in assortative mate choice, such as European badgers (Sin *et al.* 2015), should show lower invasive potential than species with disassortative mating. The development and performance of empirical tests to tackle this hypothesis will be highly informative for the process of understanding invasiveness and resolving the genetic paradox of invasive species. The potential role of MHC genes in social choices of *both* males and females expected to lead to outbreeding has never previously been addressed. We expect that the model described here will soon also be found in other species.

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Competing Financial Interests

All authors declare the absence of any competing financial interests.

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S.S. and F.-U.M. designed the study. F.-U.M. was responsible for fieldwork and contributed all samples. The wet laboratory analyses were carried out by a technical assistant and supervised by S.S. The technical assistant processed the raw data supervised by S.S. and P.S.C.S. P.S.C.S. modified and performed the bioinformatics pipeline and statistical analyses. P.S.C.S. and S.S. wrote the first draft of the manuscript. All authors contributed significantly to the final version of the manuscript.

Data accessibility

Python (bioinformatics) and R (statistics) scripts will be made available to all interested researchers upon request to PSCS (pablo.santos@uni-ulm.de). MHC genotyping and family data of all individuals investigated here are publicly available through the Dryad Digital Repository (doi:10.5061/dryad.1vb0h).

Supporting information

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

Table S1 Alignment and overall abundances of the amino acid sequences of the 22 MHC class II alleles observed in raccoons (N = 143).

Table S2 GLMM on the role of the different MHC dissimilarity and diversity indices predicting female mate choice.

Table S3 GLMM on the role of the different MHC dissimilarity and diversity indices predicting male choice of coalition partners.

Table S4 Tests for overdispersion of data points in both datasets: female choice of mates and male choice of coalition partners.

Table S5 Quality of microsatellite markers.