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HETEROZYGOSITY-FITNESS CORRELATIONS REVEALED BY NEUTRAL AND CANDIDATE GENE MARKERS IN ROE DEER FROM A LONG-TERM STUDY

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Heterozygosity-fitness correlations (HFCs) are increasingly reported but the underlying mechanisms causing HFCs are generally poorly understood. Here, we test for HFCs in roe deer (*Capreolus capreolus*) using 22 neutral microsatellites widely distributed in the genome and four microsatellites in genes that are potentially under selection. Juvenile survival was used as a proxy for individual fitness in a population that has been intensively studied for 30 years in northeastern France. For 222 juveniles, we computed two measures of genetic diversity: individual heterozygosity (*H*), and mean d^2 (relatedness of parental genomes). We found a relationship between genetic diversity and fitness both for the 22 neutral markers and two candidate genes: IGF1 (Insulin-like Growth Factor I) and NRAMP (natural resistance-associated macrophage protein). Statistical evidence and the size of genetic effects on juvenile survival were comparable to those reported for early development and cohort variation, suggesting a substantial influence of genetic components on fitness in this roe deer population. For the 22 neutral microsatellites, a correlation with fitness was revealed for mean d^2, but not for *H*, suggesting a possible outbreeding advantage. This heterosis effect could have been favored by introduction of genetically distant (Hungarian) roe deer to the population in recent times and, possibly, by the structuring of the population into distinct clans. The locus-specific correlations with fitness may be driven by growth rate advantages and resistance to diseases known to exist in the studied population. Our analyses of neutral and candidate gene markers both suggest that the observed HFCs are likely mainly due to linkage with dominant or overdominant loci that affect fitness ("local" effect) rather than to a genome-wide relationship with homozygosity due to inbreeding ("general" effect).

KEY WORDS: **Candidate genes, functional genes, heterozygosity, inbreeding, juvenile survival, microsatellites, ungulate.**

Inbreeding leads to the increased expression of recessive deleterious alleles and loss of heterozygous advantage (Charlesworth and Charlesworth 1987; Lynch and Walsh 1998), and may depress individual fitness. In recent years, individual inbreeding levels have commonly been estimated from multilocus genotype data obtained with neutral markers. This has enabled tests for correlations between fitness and genetic diversity in the wild, even in the absence of pedigree information, which is difficult to obtain in natural populations (but see Pemberton 2008). A number of studies have reported correlations between multilocus heterozygosity in noncoding DNA and fitness-related traits (Coltman et al. 1998; Coulson et al. 1999; Thelen and Allendorf 2001; Foerster et al. 2003; Hoffman et al. 2004; Markert et al. 2004; Seddon et al. 2004; Da Silva et al. 2005; Brouwer et al. 2007; Luikart et al. 2008), however, this relationship seems not to be universal (Duarte et al. 2003), and remains relatively weak in most studies (Coltman and Slate 2003; Say et al. 2004).

Three mechanisms are currently advanced to explain heterozygosity-fitness correlations (HFCs). First, some marker loci are under direct selection, with heterozygous genotypes exhibiting the highest fitness (David 1998; Hansson and Westerberg 2002). This explanation is generally excluded when using microsatellites widely distributed in the genome (i.e., located in noncoding DNA and hence hypothesized to be selectively neutral markers). However, microsatellites located in introns of genes can be used individually to investigate HFCs, given that the genes considered may be under direct selection (i.e., markers in genes). Few studies have used this approach to investigate HFCs, but increased availability of gene sequences in many species now makes feasible the genotyping of markers in genes (e.g., Vasemagi et al. 2006). From a study of an unmanaged population of Soay sheep (*Ovis aries*) Paterson et al. (1998) detected an association between Major Histocompatibility Complex variation, juvenile survival, and parasite resistance using two microsatellites in MHC genes. Coltman et al. (2001) demonstrated an association between a microsatellite polymorphism in the gamma interferon gene and resistance to gastrointestinal nematode infection in the same population of Soay sheep. Acevedo-Whitehouse et al. (2005) found heterozygosity to be negatively associated with tuberculosis infection in wild boar.

The second possible explanation is that the markers are linked with either overdominant or dominant loci influencing fitness. Under this local effect hypothesis, apparent heterozygote advantage results from genetic associations between the neutral marker loci and linked loci under selection (Hill and Robertson 1968; Otah 1971; David 1998; Hansson and Westerberg 2002).

Third, in partially inbred populations, multilocus heterozygosity at marker loci might reflect variation in the individual inbreeding level (Weir and Cockerham 1973), such that HFCs are generated as a result of effects of homozygosity at loci genome-wide (Hansson and Westerberg 2002; Slate et al. 2004); this hypothesis is sometimes termed general effect hypothesis (Weir and Cockerham 1973; David 1998).

Until recently, HFCs were often attributed to genome-wide inbreeding depression, but recent papers have highlighted the possible importance of physical linkage and local effects in such correlations. Indeed, the studies of Balloux et al. (2004) and Slate et al. (2004) reported limited power for multilocus heterozygosity to detect interindividual variance in inbreeding. This suggests that the most parsimonious explanation for many studies reporting

HFCs is a local effect rather than a general effect (Balloux et al. 2004; Slate et al. 2004; Pemberton 2004), unless the population experiences severe inbreeding events, for example through selfing, marked population structure, high levels of polygyny and/or perhaps occasional long distance dispersal (Balloux et al. 2004). Moreover, substantial linkage disequilibrium has been found in vertebrates (Reich et al. 2001; McRae et al. 2002), and the local effect hypothesis has received some support in the great reed warbler *Acrocephalus arundinaceus* (Hansson et al. 2001, 2004), and the few available studies with markers in genes (Acevedo-Whitehouse et al. 2005; Luikart et al. 2008).

Here, we investigate HFCs in a population of roe deer (*Capreolus capreolus*) intensively monitored for more than 20 years. We assess the relationship between juvenile survival, a reliable proxy for individual fitness in this population (Gaillard et al. 1998a), and genetic diversity. Individual genetic diversity was measured with the classical index of heterozygosity (H), and with the d^2 statistic (squared difference in allele length at a given microsatellite locus), introduced by Coulson et al. (1998). Numerous studies have postulated that the d^2 measure might be a better indicator of the level of outbreeding than H (Coltman et al. 1998; Coulson et al. 1998, 1999; Höglund et al. 2001; Rossiter et al. 2001; McDougall-Shackleton et al. 2005). However, others have suggested that the conditions under which d^2 performs better than H are restricted and have not been defined precisely in the wild (substructure and admixture for example, Tsitrone et al. 2001; Hedrick et al. 2001; Goudet and Keller 2002).

In this study, the influence of genetic diversity on juvenile survival was assessed while controlling for ecological and physiological variables known to affect juvenile survival in this species. The expression of HFCs may be context-dependent, being particularly strong during stressful conditions (Danzmann et al. 1988; Dudash 1990; Borsa et al. 1992; Audo and Diehl 1995; Meagher et al. 1997; Crnokrak and Roff 1999; Richardson et al. 2004; Lesbarrères et al. 2005). The roe deer study population experienced adverse climatic conditions during some springs during the course of the study (Gaillard et al. 1997), so HFCs may be strongest during these stressful periods (but see Hoffman et al. 2006).

Here, we assess genetic diversity by using multilocus heterozygosity of microsatellites in noncoding DNA, and also by using microsatellites in genes of potential interest. The two approaches have seldom been coupled. Finally this work enabled us to investigate the underlying mechanism of HFCs detected using neutral markers. Balloux et al. (2004) suggested a test of the general effect hypothesis, based on the principle that if average heterozygosity reflects f (i.e., the pedigree), then the heterozygosity of loci within an individual should be correlated. We carried out this test here using the 22 neutral microsatellite markers of our study.

Material and Methods

STUDY AREA AND POPULATION

We collected data from the roe deer population of Trois Fontaines that has been intensively monitored for 30 years (e.g., Gaillard et al. 2003). Trois Fontaines (48°43′N, 2°61′W) is a fenced forest of 1360 ha located near Saint-Dizier at the border of Marne and Haute-Marne counties in north-eastern France. The climate is continental, characterized by cold winters (mean daily temperature in January is 2°C) and hot, but not dry, summers (mean daily temperature in July is 19°C and total rainfall in July–August is 130 mm). The overstory is dominated by oak, (*Quercus* sp.; 63.5% of timber trees) and beech (*Fagus sylvatica*; 19.5%), whereas the coppices are composed primarily of hornbeam (*Carpinus betulus*; 70%). The understory is dominated by ivy (*Hedera helix*) and bramble (*Rubus* sp.), which are the principal and preferred foods of roe deer (Duncan et al. 1998). The soils at Trois Fontaines are fertile and the forests are highly productive (long-term average of 5.92 m^3 of wood produced/ha/year, data from Inventaire National Forestier). Trois Fontaines is considered good habitat given our current knowledge of food habits of roe deer (Duncan et al. 1998).

The roe deer population size is controlled by yearly removals. About 10 days of capture by drive netting have been organized each winter since 1976, permitting the sampling of the entire study area every year. The roe deer population in Trois Fontaines was maintained at 200–250 individuals $>$ 1 year old in March from 1977 to 2000 by intensive culling (Gaillard et al. 2003), and is highly productive, with all 2-year-old females breeding (Gaillard et al. 1998b). Annual survival rates average 0.82 and 0.93 for adult males and females, respectively (Gaillard et al. 1993b), and the annual finite rate of increase (λ) averages 1.37 which is close to the maximum for roe deer (i.e., r-max sensu Caughley 1977). From 1985 onwards, newborn fawns were searched for during the fawning season (May-June, Gaillard et al. 1993c). Fawns were captured by hand and marked. After a fawn was marked with colored and numbered ear-tags, it was weighed to the nearest 50 g, and its age (based on umbilicus features and behavior, Jullien et al. 1992) and sex were determined. The mean age of fawns at marking was 4.8 days during the study years (range = 3.1 days in 1998 to 6.6 days in 1995). The mean Julian birth date was 138 (range of 136 in 1995 to 140 in 1999). Sometimes, the mother was identified during the marking, but on too few occasions to allow us to consider maternal characteristics in the HFCs analyses. From 1994 onwards, ear tissue was taken from each fawn during the marking and preserved in 100% ethanol for the genetic analyses.

JUVENILE SURVIVAL

Juvenile survival of fawns over their first summer was used as a proxy for fitness because it is the most variable demographic

parameter in roe deer population dynamics (Gaillard et al. 1998a). Observed yearly changes in fawn survival had the largest contribution to the observed variation in the population growth rate (Gaillard et al. 1998a), which corresponds to mean individual fitness (see e.g., Caswell 2001). This indicates that summer survival of fawns is indeed the key parameter driving female fitness. We used the winter capture data to assess whether a given fawn survived or not over its first summer. All fawns marked as newborns and captured at least once in subsequent winters were considered to have survived. To reliably assess the survival of fawns from the winter capture data, we did not consider fawns born after 2000, as this meant that there were at least five occasions (winters) to catch surviving fawns from all cohorts. In other words, a fawn born in 2000 that was not recaught in any of the five subsequent winters was considered to have died. This is justified in view of the high capture probability of roe deer during winter in this site (around 0.65 for the first winter and about 0.50 thereafter, Gaillard et al. 1997, 2003), which means that the probability of a fawn being alive but not caught in any of its first 5 winters is less than 0.03. We have shown previously that such estimates of individual survival lead to yearly estimates very close to those obtained from Capture–Mark–Recapture (CMR) modeling (Gaillard et al. 1998b; Pettorelli et al. 2005).

PHENOTYPIC QUALITY

We measured the phenotypic quality of newborn fawns by an index of early body development. We used the residual values from the regression of fawn body mass at capture on age for fawns captured when less than 20 days of age (see Gaillard et al. 1993a for further details).

GENETIC DIVERSITY

Microsatellites distributed across the genome

Twenty-two putatively neutral microsatellites widely distributed in the genome were used to genotype samples. The loci were all dinucleotide loci, hence they are expected to follow expectations of the SMM. PCR amplification conditions in *C. capreolus* are described, for ROE9, ROE6, ROE1, and ROE8 in Fickel and Reinsch (2000), for IDVGA29, CSSM39, IDVGA8, BM1706, HUJ1177, OarFCB304, BM848, NVHRT48, CSSM41, BM757, and CSSM43 in Galan et al. (2003), and for MAF70, ILST011, ILST030, BM1818, BMS119, SR-CRSP1, and BMC1009 in Vial et al. (2003).

Two genetic diversity measures were calculated: H_S, a standardized measure of individual heterozygosity, which is the proportion of heterozygous loci/mean heterozygosity of typed loci, because all individuals were not typed at all the marker loci (as in Coltman et al. 1999). We also used one measure derived from mean d^2, i.e., the squared distance in repeat units between the two alleles of an individual at a given microsatellite locus, averaged over all loci at which an individual was scored (Coulson et al. 1998).

Assuming that microsatellites evolve under the stepwise mutation model (Valdes et al. 1993), mean d^2 provides a measure of the genetic distance between parental gamete genomes. Mean d^2 refers to the mean genome-wide d^2 and thus has been considered a measure of an individual's overall level of outbreeding (d^2 for a given locus is a locus-specific measure to test for local effects). That is, individuals with alleles at a given locus that differ the most in the number of repeat units are presumed to have higher levels of outbreeding and the parental alleles have more ancestral coalescence times. In contrast, measures of H should better translate levels of inbreeding (Coulson et al. 1999). The logarithmic transformation was used to reduce the pronounced skew of the predictor variable d^2 and therefore reduce the influence of extreme values: mean d^2 was then measured by $LD = \log(\text{mean } d^2 + 1)$.

Intron-based microsatellites

In the literature, we identified 30 microsatellites (Table 1) that are located in the introns of genes from cattle, sheep, and goats. Each microsatellite (primer pair) was tested for amplification and polymorphism in roe deer. Four showed a clear profile and were polymorphic (IGF1, NRAMP1, MMP9, and KRT2; Table 2).

PCR amplifications were conducted in a 12 μl volume containing approximately 20 ng of template DNA, 0.1 mM of each dNTP, 0.6 μM of each primer (with a fluorescent label on the forward primer from Proligo, although we used NED colored primers from Applied Biosystems), 2.5 mM $MgCl_2$, 0.6 U Amplitaq Gold Polymerase (Applied Biosystems, Foster City, CA), and 1 × Taq Buffer (according to the manufacturers specification; Applied Biosystems). Amplifications were performed in GeneAmp PCR System 2400 (Applied Biosystems), with the following cycling conditions: 10 min at 95°C, n cycles composed of 30 sec denaturing at 95°C, 30 sec annealing at Tm, 30 sec extension at 72°C, and 7 min at 72°C to ensure complete extension (see references in Tables 1 and 2 for Tm and n). Amplified fragments were then loaded on 5% Long Ranger polyacrylamide gels and electrophoresis was run for 3 h on an automated sequencer ABI 377™ (Applied Biosystems). Microsatellite patterns were examined with Genotyper 2.0 (Applied Biosystems).

We also used the two measures of individual genetic diversity (H and d^2) calculated for each locus independently. We thus obtained a measure of individual heterozygosity (H) at each locus (H_{igf1}, H_{nramp1}, H_{krt2}, H_{mmp9}), as well as a d^2 value (LD_{igf1}, LD_{nramp1}, LD_{krt2}, LD_{mmp9}).

For both types of microsatellites (neutral and candidate selected loci), tests for deviation from Hardy–Weinberg proportions and linkage disequilibrium were performed using GENEPOP ver. 3.3 (Raymond and Rousset 1995), considering all cohorts together and also for each cohort separately.

Table 1. Characteristics of microsatellites widely distributed in the genome. PCR amplification conditions in *Capreolus capreolus* are described, for ROE9, ROE6, ROE1, and ROE8 in Fickel and Reinsch (2000), for IDVGA29, CSSM39, IDVGA8, BM1706, HUJ1177, OarFCB304, BM848, NVHRT48, CSSM41, BM757, CSSM43 in Galan et al. (2003), and for MAF70, ILST011, ILST030, BM1818, BMS119, SR-CRSP1, BMC1009 in Vial et al. (2003).

Locus name	Localization (Chromosome)	Na	Size range (bp)	Ho	He	HWE	LD
MAF70	Chromosome 4 (bovine)	10	129–173	0.72	0.70	ns	86.25
ROE9	Unknown	3	182–186	0.49	0.48	ns	3.19
ILST011	Chromosome 9 (ovine)	6	274–284	0.79	0.77	ns	25.74
ROE6	Unknown	8	91–111	0.68	0.73	ns	103.41
ROE1	Unknown	3	132–136	0.39	0.33	ns	1.55
ILST030	Chromosome 2 (bovine)	8	157–179	0.56	0.70	«first» cohort P=0.0011 ns (other cohorts)	33.79
BM1818	Chromosome 20 (ovine)	10	249–267	0.81	0.79	ns	45.25
ROE8	Unknown	9	60–90	0.75	0.75	ns	74.12
BMS119	Chromosome 1 (ovine)	3	101–107	0.15	0.19	ns	2.31
SR-CRSP1	Unknown	8	134–160	0.83	0.78	ns	153.19
BMC1009	Chromosome 3 (ovine)	6	280–290	0.69	0.71	ns	18.34
IDVGA29	Chromosome 28 (bovine)	2	141–147	0.33	0.33	ns	11.66
CSSM39	Chromosome 1 (ovine)	6	175–185	0.79	0.77	ns	25.25
IDVGA8	Chromosome 11 (bovine)	6	208–224	0.73	0.73	ns	21.85
BM1706	Chromosome 16 (bovine)	8	235–251	0.70	0.72	ns	38.53
OarFCB304	Chromosome 19 (ovine)	19	152–183	0.79	0.80	ns	73.61
HUJ1177	Chromosome 3 (ovine)	10	199–223	0.76	0.79	ns	34.52
BM848	Chromosome 15 (ovine)	10	348–368	0.77	0.77	ns	28.81
NVHRT48	Unknown	3	86–90	0.45	0.42	ns	2.29
CSSM41	Chromosome 19 (ovine)	3	120–124	0.49	0.50	ns	4.08
BM757	Chromosome 9 (ovine)	9	172–204	0.81	0.81	ns	216.08
CSSM43	Chromosome 27 (bovine)	5	238–246	0.75	0.7	ns	8.54

Na, number of alleles; Ho, observed Heterozygosity; He, unbiased expected heterozygosity (Nei 1978); HWE, significance level of deviation from expected Hardy–Weinberg genotype frequencies calculated with Fisher's exact test (Raymond and Rousset 1995); LD, mean d^2.

TEST OF GENERAL VS. LOCAL EFFECTS

We used the test proposed by Balloux et al. (2004) to test the general effect hypothesis. We randomly divided our sample of 22 loci into two groups of 11 loci, and asked whether, across individuals, the heterozygosity (measured by H and mean d^2) of the first group was correlated with the heterozygosity (H and d^2) of the second group. Then, by resampling the 22 loci we repeated the procedure 100 times (as suggested in Balloux et al. 2004) to obtain a mean and a standard error for the correlation.

STATISTICAL MODELS AND ANALYSES

All analyses were performed with the *R* package, (R 2.6.1, R Development Core Team 2007). We used logistic regression models (logit link and binomial distribution) to model juvenile survival

Table 2. Characteristics of microsatellites located in genes and typed in roe deer.

	Na	Size range (bp)	Tm	n	Ho	He	HWE	LD
NRAMP1	14	209–239	65–55[1]	10+35	0.85	0.84	ns	128.1
KRT2	7	135–148	55	40	0.71	0.71	ns	23.33
IGF1	4	120–128	58	40	0.45	0.44	ns	2.15
MMP9	5	185–193	58	40	0.65	0.63	ns	15.54

Na, number of alleles; Tm, annealing temperature; *n*, number of cycles; Ho, observed Heterozygosity; He, unbiased expected heterozygosity (Nei 1978); HWE, significance level of deviation in expected genotype frequencies from Hardy–Weinberg expectation calculated by Fisher's exact test (Raymond and Rousset 1995); LD, mean d^2.

[1]Starting from the first indicated temperature (left), the annealing temperature is decreased by 1° C per cycle for the first 10 cycles, and is subsequently stabilized for 35 cycles at the second indicated temperature.

(McCullagh and Nelder 1989). We included yearly survival estimated using CMR-analyses to describe quantitative variation among cohorts. We did not include sex as a factor in our analyses because fawn survival has been reported to be similar in the two sexes in this population (Gaillard et al. 1998b), as well as in other roe deer populations (Gaillard et al. 1998a; Pettorelli et al. 2005).

First, a minimal nongenetic model (Paterson et al. 1998; Coltman et al. 2001) was built to describe fawn survival in relation to body development, cohort survival, and the interactions between these terms. Subsequently, genetic terms were added to the selected nongenetic model. We also tested for genetic effects without accounting for body development. Indices of genetic diversity obtained from neutral microsatellites widely distributed in the genome (H and d^2) and from microsatellites in genes (H_{igf1}, H_{nramp1}, H_{krt2}, H_{mmp9} and LD_{igf1}, LD_{nramp1}, LD_{krt2}, LD_{mmp9}) were fitted separately.

Our aim was to identify the best statistical model(s) in terms of predicting juvenile survival. For this kind of model selection, there are many available approaches, in particular those based on selection criteria such as the Akaike Information Criterion (*AIC*) (e.g., Burnham and Anderson 2002; Johnson and Omland 2004) and those based on a quantitative measure of prediction power estimated through cross-validation (e.g., Harrell 2001; Hastie et al. 2001). As *AIC* is widely used in statistical analyses of biological processes (Burnham and Anderson 2002), we calculated the *AIC* scores corrected for small sample size (*AICc*) for each model tested. We also calculated *AICc* weights to measure the relative likelihood of a given model among the set of models tested (Burnham and Anderson 2002). In our analysis based on *AIC*, we restricted the sample size to the individuals measured for all traits so as to have the same sample size.

Measures based on cross-validation have the advantage of providing a robust measure of the predictive ability of a model (e.g., Harrell 2001; Hastie et al. 2001). Moreover, such measures can be compared across subsets of data with different sample sizes, something that is not possible with *AIC*. We used *K*-fold cross-validation for comparing the best models: observations were randomly divided into K sets with an approximately equal number of observations, $K - 1$ sets being used for estimation of model parameters and the last set being used for model validation (i.e., calculating the predictive performance of the model). The prediction criterion was averaged over the K different choices of validation set. Values of K between 5 and 10 are recommended (Hastie et al. 2001) and we therefore investigated both values. For each model and value of K, 500 random partitions were used to estimate the measure of model predictive ability, and the bias-corrected value for the predictive criterion was used (Davison and Hinkley 1997). The two values 5 and 10 for K gave the same results hence, only the results for fivefold validations are presented here. As a predictive criterion, we used the percentage of misclassified observations (that is, individuals with a predicted value below a given threshold are expected to die and those with a predicted value above the threshold are expected to survive). We used a threshold value of 0.5, which results in a criterion of 0.41 for the constant model, the average survival rate in our sample. Using a threshold of 0.5 assumes an equal cost of misclassifying fawns that died or survived.

Results

Analyses were performed on 222 fawns captured between 1994 and 2000. Ninety-three percent of these animals were typed at 19 to 22 microsatellites. As in other studies (e.g., Coltman et al. 1999) only the genotyped loci were used in standardized estimates of individual H (Table 3). Two hundred and nineteen fawns were successfully typed at the four microsatellites located in genes (Table 3).

NONGENETIC MODEL

Of the three possible models to predict juvenile survival including only the nongenetic terms (in addition to the constant model M0), the model M3 including the variables cohort survival and body development was retained (being >7.6 times more supported than the model including only one of these variables and >120 times more supported than the constant model based on *AICc* weights, Table 4). As expected, the probability that a given fawn survived increased with increasing cohort survival (3.06 ± 1.25) and increasing body development (0.74 ± 0.30). Adding the multiplicative term cohort survival $\times$ body development to M3 did not improve prediction of fawn survival rates (2.8 times less support for the multiplicative model).

Table 3. Mean yearly fawn survival and number of fawns analyzed each year for neutral and candidate adaptive microsatellites (Trois Fontaines roe deer population, France). Fawn survival was estimated by Capture–Mark–Recapture methods (see Gaillard et al. 1997).

Year	1994	1995	1996	1997	1998	1999	2000
Fawn survival	0.50	0.63	0.35	0.42	0.50	0.25	0.41
Neutral	7	19	45	41	30	43	37
Candidate adaptive	6	19	46	41	30	40	37

Table 4. Predictive models of juvenile survival using microsatellites randomly distributed in the genome. For each model, the difference between the *AICc* score of a given model and that of the best model (Δ*AICc*), plus *AICc* weights are provided and the misclassification rate (MisR) is estimated by cross-validation (fivefold partition; Harrell 2001). *AICc* values are not comparable to those in Table 5 due to different sample sizes, but misclassification rates are. The best models occur in bold, *K* corresponds to the number of different partitions involved in the analysis (see text for further details), "×" represents an interaction. S, cohort survival; BD, body development; I, indicates that the variable is included in the model.

Nongenetic models	S	BD	Δ*AICc*	*AICc* weight	MisR CV (*K*=5)	Estimates of genetic effects (SE, *P*-values)	Estimates (SE, *P*-values) of other effects (S+BD)
M0			14.56	0.000	0.4099		
M1	I		9.13	0.006	0.3989		
M2		I	8.92	0.006	0.4215		
M3	**I**	**I**	**4.86**	**0.049**	**0.3738**		**3.06 (1.25; 0.014); 0.742 (0.301; 0.014)**
M4	I	I	6.93	0.017	0.3771		
(M3+S×BD)							
S+genetic effect	H	LD					
	I		11.02	0.002	0.4055	−0.358 (0.897; 0.69)	
		I	6.62	0.02	0.3811	1.35 (0.653; 0.038)	
S+BD+genetic effect	H	LD					
	I		7.56	0.013	0.3866	−0.560 (0.911; 0.54)	
		I	1.78	0.228	0.3541	1.46 (0.664; 0.028)	3.24 (1.27; 0.011); 0.783 (0.304; 0.010)
H×S	I		8.22	0.009	0.3992	5.07 (7.76; 0.51)	
LD×S		**I**	**0**	**0.555**	**0.3601**	**LD×S: 10.9 (5.64; 0.053); LD 1.55 (0.665; 0.020)**	**3.35 (1.30; 0.0098); 0.779 (0.306; 0.011)**
H×BD	I		8.64	0.007	0.3855	0.211 (1.88; 0.91)	
LD×BD		I	3.70	0.087	0.3485	0.683 (1.65; 0.68)	

NEUTRAL MICROSATELLITES GENOME WIDE

Analyses performed on the 22 microsatellites widely distributed in the genome revealed no significant deviations from Hardy–Weinberg proportions, except for the locus ILST030 (Table 1). For this locus a deficit of heterozygotes was detected, but only in the first cohort ($P = 0.001$), perhaps due to random chance or perhaps a low-frequency nonamplifying (null) allele. There was no evidence for gametic disequilibrium between any loci ($P > 0.05$ for all pairs of loci). Mean heterozygosity was 0.645 ± 0.009, and ranged from 0.36 to 0.95 among loci.

We built four models by adding separately each of the two genetic terms (H and d^2) to the chosen nongenetic model (M3) or simply to the model with only a cohort effect (M1). The addition of d^2 to both the chosen nongenetic model and the simple cohort effect model improved the nongenetic minimal model (being 3.5 and 4.7 times more supported, respectively, than nongenetic models based on *AICc* weights, Table 4). As predicted, the probability of fawn survival increased with increasing d^2 (1.46 ± 0.66). On the other hand, H did not influence juvenile survival (being 2.6 and 3.9 times less supported, respectively, than nongenetic models based on *AICc* weights, Table 4). The model including the interaction terms d^2: cohort survival was somewhat better at predicting fawn survival (being 2.4 times more supported than the additive model based on *AICc* weights, Table 4), indicating that this heterozygous advantage was stronger during "favorable" years (slope increase of 0.28 ± 0.15 on a logit scale). Model ranking based on cross-validated predictive values was similar to the *AICc* ranking, except for the model with the interaction between *LD* and body development that got some support in the former case. Interestingly, when using standardized variables to account for differences in units, the effect sizes (i.e., slopes) of early body development and LD were remarkably similar (0.38 and 0.34, respectively), indicating that phenotypic and genetic measures of individual quality had similar degrees of influence on juvenile survival (Fig. 1).

ANALYSES WITH MICROSATELLITES IN GENES

The genotype frequencies for the four microsatellites in genes did not deviate significantly from Hardy–Weinberg proportions (Table 2). There was no evidence for gametic disequilibrium between any loci ($P > 0.05$ for all pairs of loci).

We tested for an association between each locus characterized by a given H and a given LD (H_{igf1}, H_{nramp1}, H_{krt2}, H_{mmp9}, and LD_{igf1}, LD_{nramp1}, LD_{krt2}, LD_{mmp9}) and juvenile survival by fitting

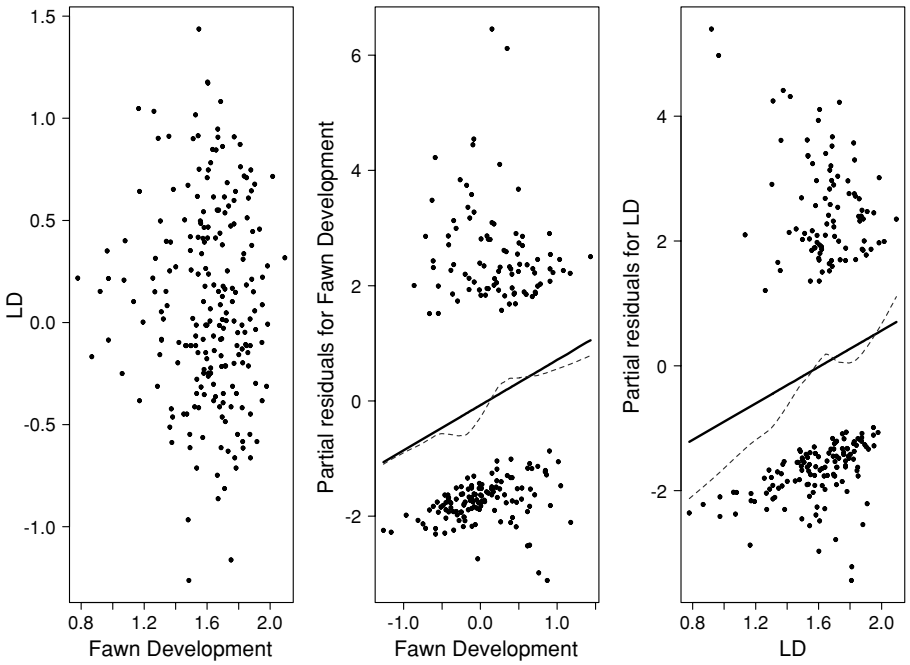

Figure 1. Graphical display of the influence of the phenotypic trait (early fawn development) and of the genetic trait (*LD*) on juvenile survival of roe deer. The left panel shows the absence of correlation between the phenotypic and the genetic traits. The other panels show that, in terms of effect size, early fawn development and *LD* have very similar positive influences on juvenile survival (after juvenile survival has been corrected for the influence of LD and yearly survival in the first case, and by body development and yearly survival in the second). The dashed line represents a nonparametric fit to the partial residuals.

each term separately to the nongenetic minimal model (M3) and to the model with a cohort effect only.

Models containing the genetic terms H_{ig} (0.564 ± 0.293 and 0.531 ± 0.288 with or without the effect of body development, respectively) were 2.3 and 2.0 times more supported, respectively, than nongenetic models based on *AICc* weights (Table 5). For H_{ig} no model with interaction terms improved the predictions, whereas for LD_{nramp1} the model including the LD_{nramp1}: body development interaction term gave the best prediction, indicating that this heterozygous advantage was stronger for juveniles with a higher body development (LD_{lramp1}: body development: 0.441 ± 0.155) (Table 5).

Interestingly, when we accounted for the effects of both cohort survival and body development, the effect size of *LD* was positive in all of the four microsatellites in genes although only weakly statistically supported for the additive effect of locus NRAMP (Table 5), supporting the hypothesis of a general positive effect of *LD* in these microsatellites in genes on juvenile survival. Note, however, that the support for the effect of H_{ig} and LD_{ig} in terms of predictive value was strong (Table 5)

"LOCAL" VERSUS "GENERAL" EFFECT HYPOTHESIS

To investigate the underlying mechanism of the HFC revealed by the set of neutral markers used, we considered the 22 microsatellites randomly distributed in the genome individually, fitting *LD* and *H* individual values for each microsatellite to the nongenetic minimal model (M3). Of the 22 models with *LD* values and the 22 with *H* values (based on each individual locus), none was better than the nongenetic minimal model (Fig. 2).

To apply the test of the general effect hypothesis (Balloux et al. 2004), we tested for a heterozygosity correlation between two random sets of 11 loci. We obtained, for the *H*–*H* correlation, an estimated mean *r* of 0.0341 (SD = 0.053) and for the $d^2 - d^2$ correlation, an estimated mean *r* of 0.005 (SD = 0.052).

Discussion

We found a relationship between genetic diversity and fitness both for the neutral markers and two candidate genes: IGF1 and NRAMP. The size of genetic effects on juvenile survival was comparable to those reported for early development and cohort variation, suggesting a substantial influence of genetic components on fitness in this roe deer population. For the neutral microsatellites, a correlation with fitness was revealed for mean d^2, but not for H, suggesting a possible outbreeding advantage. Our analyses of neutral and candidate gene markers both suggest that the observed HFCs are likely mainly due to linkage with dominant or overdominant loci that affect fitness ("local" effect) rather than to a global relationship with homozygosity due to inbreeding ("general" effect). In the following, we discuss our measure of statistical

Table 5. Predictive models of juvenile survival considering microsatellites in genes. For each model, the difference between the *AICc* score of a given model and that of the best model (Δ*AICc*), *AICc* weights are provided and the misclassification rate is estimated by cross-validation (Harrell 2001). *AICc* values are not comparable to those in Table 4 due to different sample sizes, but misclassification rates are. The best models occur in bold. S, cohort survival; BD, body developement; I, indicates that the variable is included in the model.

Model	S	BD			Δ*AICc*	*AICc* weight	MisR CV (K=5)	Estimate genetic effect (SE, *P*-value)	Estimate other effect (SE, *P*-value)
M0					15.01	0.000	0.4037		
M1	I				8.93	0.005	0.3922		3.527 (1.255; 0.005)
M2		I			8.89	0.005	0.4102		
M3	**I**	**I**			**4.44**	**0.045**	**0.3744**		**3.216 (1.273; 0.012); 0.767 (0.306; 0.012)**
S	H_{ig}	H_{mm}	H_{kr}	H_{nramp}					
	I				**7.57**	**0.009**	**0.3994**	**0.531 (0.288; 0.065)**	
		I			10.98	0.002	0.3982	0.0089 (0.299; 0.976)	
			I		10.98	0.002	0.3978	−0.012 (0.308; 0.969)	
				I	10.83	0.002	0.3958	−0.157 (0.398; 0.693)	
	LD_{ig}	LD_{mm}	LD_{kr}	LD_{nramp}					
	I				8.59	0.006	0.4043	0.258 (0.167; 0.123)	
		I			9.70	0.003	0.3774	0.105 (0.093; 0.257)	
			I		10.51	0.002	0.4149	0.058 (0.084; 0.493)	
				I	10.97	0.002	04007	−0.010 (0.071; 0.890)	
S+BD	H_{ig}	H_{mm}	H_{kr}	H_{nramp}					
	I				**2.79**	**0.103**	**0.3480**	**0.564 (0.293; 0.054)**	
		I			6.50	0.016	0.3659	0.0381 (0.304; 0.900)	
			I		6.50	0.016	0.3833	−0.032 (0.313; 0.919)	
				I	6.19	0.016	0.3749	−0.230 (0.404; 0.570)	
	LD_{ig}	LD_{mm}	LD_{kr}	LD_{nramp}					
	I				4.11	0.053	0.3743	0.265 (0.172; 0.123)	2.784 (1.307; 0.033); 0.773 (0.308; 0.012)
		I			4.97	0.035	0.3742	0.117 (0.094; 0.215)	
			I		6.09	0.020	0.3623	0.056 (0.085; 0.514)	
				I	6.51	0.016	0.3788	0.0040 (0.0073; 0.956)	
× BD	H_{ig}	H_{mm}	H_{kr}	H_{nramp}					
	I				4.82	0.037	0.3558		
		I			8.22	0.007	0.3895		
			I		8.57	0.006	0.3848		
				I	7.41	0.010	0.3777		
× S	I				4.62	0.041	0.3546		
				I	7.46	0.010	0.3912		
× BD	LD_{ig}	LD_{mm}	LD_{kr}	LD_{nramp}					
	I				6.20	0.019	0.3508	−0.026 (0.34; 0.94)	
		I			3.64	0.068	0.3765	0.379 (0.209; 0.069)	
			I		8.18	0.007	0.3666	0.0073 (0.184; 0.968)	
				I	**0**	**0.417**	**0.3688**	**0.441 (0.155; 0.0045)**	**3.28 (1.30; 0.012); 0.89 (0.33; 0.0066)**
× S	I				7.87	0.008	0.3515		
				I	7.87	0.008	0.3963		

evidence of genetic effects, the potential problems of not accounting for the identity of parents, how maternal effects could account for the HFCs we reported, the HFCs using both microsatellites in genes and microsatellites distributed across the genome, and the possible underlying mechanisms of these HFCs.

Measures of statistical evidence

We used here as a measure of statistical evidence two criteria based on the predictive ability of models—one based on information and statistical theory, *AICc*, and an empirical one based on cross-validation. The two approaches gave consistent

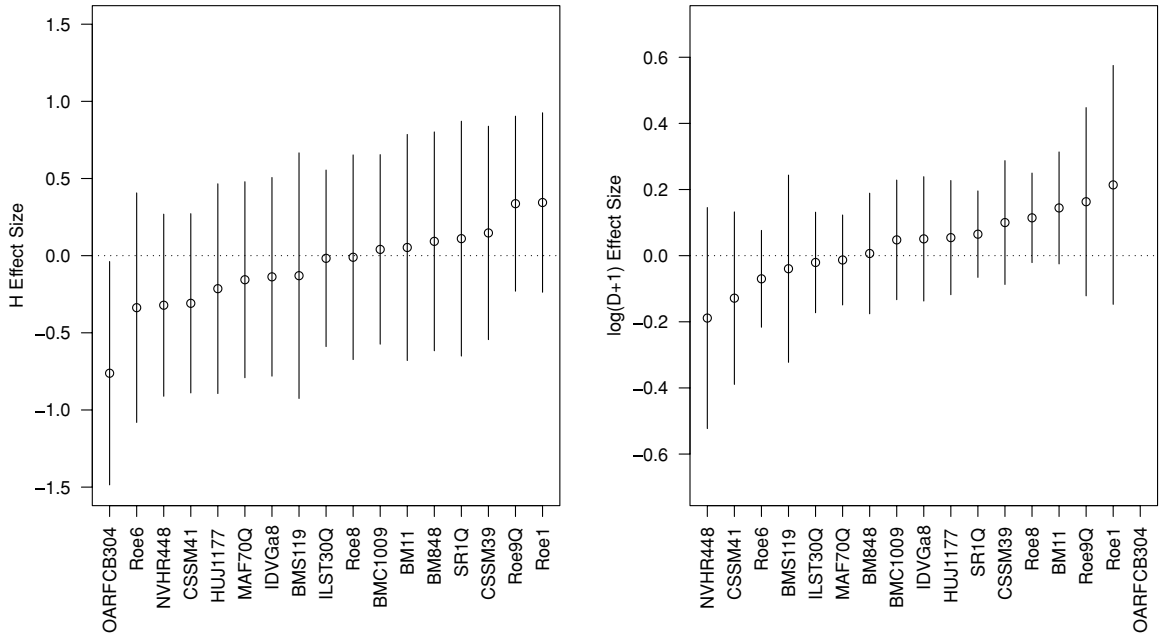

Figure 2. Effect size of the genetic influence for the various loci (*H* on the left panel and *LD* on the right panel). Note the clear difference of effect size between the two genetic measures: no effect for *H*, positive effect size at most loci for *LD*.

results, emphasizing the robustness of our model selection. For microsatellites in the genome, the best genetic model had an *AICc* nearly five units lower than the best nongenetic model, whereas for microsatellites in genes, the difference was about four. Differences larger than two are usually taken as evidence of significant support for one model over a competing model, with a difference of four or more being considered as strong evidence of one model outperforming another model (e.g., Burnham and Anderson 2002). Significance tests were not used to select models and the problem of multiple testing is therefore not an issue. We nevertheless provided confidence intervals to emphasize the uncertainty in effect size, but this uncertainty was not larger than for the effect of body condition—reducing substantially this uncertainty would require very large sample size (i.e., thousands of individuals). One could argue that the relatively large number of models considered could lead to a problem of selection bias (Zuchini 2000), but the *AICc* weights of the genetic models were an order of magnitude larger than the nongenetic models, providing quite strong evidence of a role of genetic variation both within and outside coding regions in influencing fitness.

Potential problems of not controlling for the identity of parents

With the absence of paternity data, we lacked information on how many males sired the 222 fawns included in our analyses. However, roe deer are only weakly polygynous. Male roe deer defend small and stable territories for half of the year, do not increase home range size during the rut, and do not defend harems. Accordingly, a recent study in a Swedish population of roe deer showed that the variance in male reproductive success was rather low (Vanpé et al. 2008). It is thus unlikely that the majority of fawns could have been sired by only a few males.

The situation was different for mothers. Although we did not know the mother of most fawns included in our analyses (we could not account for possible pseudoreplication across mothers in the statistical analyses), we knew the mother of 75 of 222 fawns. These 75 fawns were produced by 37 different mothers, indicating that quite a large number of females produced fawns. From this restricted dataset, we did not find any difference in results obtained with or without accounting for pseudoreplication (results not shown), likely as a consequence of the weak amount of pseudoreplication (average of 2.03 fawns per mother, from a minimum of 1 for 17 females to a maximum of 7 for one female). At Trois Fontaines, virtually all females older than one year of age produced twins (Gaillard et al. 1998b) so we can expect the fawns we sampled corresponded to a large sample of mothers of varying performance.

Maternal effects

Roe deer fawn survival is highly variable among and within cohorts and depends on a large range of factors (Gaillard et al. 1998a for a review). The among-cohort variation in fawn survival is linked to climatic conditions during spring-summer through its impact on plant quality (Gaillard et al. 1997). For fawns born in a given cohort, early body development is likely to shape individual survival. We found, as expected, that heavy fawns at a given age survived better than light fawns. A better ability to regulate temperature for maintaining homeothermy when facing very

hot or very wet weather, or better resistance to infectious agents, could account for this higher survival advantage of heavier fawns, which has often been reported in populations of large herbivores (Gaillard et al. 2000 for a review). Heavy fawns were likely produced by heavy mothers (Clutton-Brock 1991), so phenotypic quality of mothers was expected to partially account for the positive influence of fawn body development on their survival. However, most within-cohort variation in fawn survival was shaped by spatial heterogeneities in home range composition (McLoughlin et al. 2007), which accounted for most among-female variation in the number of offspring raised during lifetime. Although we did not have genetic information on mothers, their individual heterozygosity might have influenced offspring survival. As reported by Richardson et al. (2004) on the Seychelles warbler, a positive influence of mother heterozygosity on offspring survival should not necessarily pass through fawn heterozygosity (interestingly, fawn heterozygosity tended to have a negative effect on their survival in our study, [Richardson et al. 2004], but may rather indicate a general maternal quality). Thus a set of maternal characteristics including phenotype (e.g., body mass), genetics (e.g., heterozygosity), and home range quality were the most likely factors shaping within-cohort variation in early survival of fawns.

HFCs using microsatellites in genes

Our predictive models of survival showed that the survival probability was better for heterozygous juveniles at the NRAMP locus (for the d^2 value) and the IGF1 locus (for the H value), which were two of the four microsatellites in genes. In addition, higher d^2 tended to be associated with higher juvenile survival in the two other microsatellites in genes studied here. The heterozygote advantage observed at these loci might be explained by direct selection on IGF1 and NRAMP, as both play important functions in early stages of life. Indeed, IGF1, or Insulin-like Growth Factor I (a polypeptide protein hormone), is thought to be a primary growth factor required for early juvenile or child development, and IGF-I expression is required for achieving maximal growth (Gelwane et al. 2007). IGF-I also influences the proliferation and the differentiation of cells (Baker et al. 1993; Powell-Braxton et al. 1993; Furstenberger and Senn 2002). NRAMP (natural resistance-associated macrophage protein) is an integral membrane protein expressed exclusively in immune system cells and may play a role in resistance to microbial infection (Govoni and Gros 1998). The two candidate genes not statistically associated with survival were MMP9 and KRT2. KRT2 codes for keratin fibres in hair, is a candidate gene for wool fiber quality in domestic sheep, and could play a role in insulation and thermoregulation (McLaren et al. 1997). The gene MMP9 (matrix metalloproteinase 9) is involved in the breakdown of the extracellular matrix in normal physiological processes such as embryonic development, as well as in prevention of diseases such as COPD (chronic obstructive pulmonary disease), arthritis, and metastasis (Turner et al. 2000; Nutt et al. 2003).

The HFC trend occurred for d^2 at all four loci in genes, suggesting an outbreeding advantage (where individuals with more divergent allele lengths have higher survival). For the IGF1 locus, the significant association concerned H, suggesting selection against homozygotes.

HFCs using microsatellites distributed across the genome

The 22 microsatellites widely distributed across the genome have a well-known chromosomal location (Table 1), which is highly conserved across divergent ungulate taxa (see the Roslin Institute (United Kingdom) at http://www.thearkdb.org/ or Texas A&M University (USA) at http://texas.thearkdb.org/). We found a positive association between genetic diversity and juvenile survival when using d^2 with these markers, but not when using H. Hence, these neutral markers suggested a possible survival advantage for heterozygous individuals that are more outbred.

We expected that the relationship between genetic diversity and survival would be particularly strong during stressful environmental conditions (Danzmann et al. 1988; Dudash 1990; Borsa et al. 1992; Audo and Diehl 1995; Meagher et al. 1997; Crnokrak and Roff 1999; Richardson et al. 2004, Lesbarrères et al. 2005). Conversely, the correlation between d^2 and juvenile survival was found to be particularly strong during what we defined as "good" (high survival) years. It seems that genetic diversity does not provide sufficient advantage to counteract the factors inducing higher juvenile mortality during the years 1996 and 1999 in our population. Instead, differential occurrence of preferred plant species within the maternal home range could be the decisive factor for shaping fawn survival during bad years, as found in another roe deer population (Pettorelli et al. 2005). Interestingly, in support of our findings, Hoffman et al. (2006) reported for Antarctic fur seals pups, that dying from starvation is unlikely to be influenced by genetic factors.

How can we explain the association between a component of individual fitness and d^2 indices in this study (see also Coltman et al. 1998; Coulson et al. 1998, 1999; Rossiter et al. 2001; Höglund et al. 2001), but not with the H index (see Tsitrone et al. 2001; Hedrick et al. 2001; Goudet and Keller 2002; Slate and Pemberton 2002)? We can envisage three hypotheses to explain this result: (1) the possibility that a local microgeographic substructure exists in the population leading to heterosis in matings between-subgroups, (2) the effects of admixture (heterosis) through the introduction of unrelated deer carried out in the recent past, or (3) possible immigration of individuals through the fence from the surrounding area.

Local microgeographic substructure does occur in the social organization of roe deer populations and limited postnatal

dispersal has been documented in both sexes in roe deer populations (Linnell et al. 1998). Roe deer are extremely sedentary (Strandgaard 1972) and a structure of clans among related females has been reported in roe deer populations at densities similar to that observed at Trois Fontaines (Kurt 1968; Ellenberg 1978). Moreover several studies on ungulate populations, which generally exhibit female philopatry, have revealed fine-scale genetic structure (Matthews and Porter 1993; Petit et al. 1997; Coltman et al. 2003; Nussey et al. 2005), even at a small geographic scale (Coltman et al. 2003). Individuals of a given subgroup should be genetically closer to each other than to individuals from other subgroups, because of a high level of philopatry in both roe deer sexes (Linnell et al. 1998). Occasional gene flow between subgroups could perhaps lead to a slight outbreeding and heterosis effect among individuals. However, local substructure alone seems unlikely to explain how divergent alleles and microsatellites of markedly different length (d^2) generated the observed HFC.

Roe deer from a distant population in Hungary (>1000 km east of our study population), as well as some roe deer from the Côte d'Or department in France (>150 km south of our study population), were introduced into our study population in 1974–1975 to avoid putative negative consequences of inbreeding in this closed population. Hence this introduction of likely divergent alleles (Hungarian roe deer are phylogeographically quite different from French populations, Vernesi et al. 2002), possibly combined with a substructure effect, could have lead to an outbreeding advantage. An influx of alternative alleles through occasional immigration from adjacent populations is unlikely because the fence around the study site enclosure is regularly checked and any holes are quickly repaired; furthermore population density of roe deer outside has been consistently lower than inside the reserve so that if any exchanges ever occur, emigration rather than immigration should be expected.

Underlying mechanism of HFCs

The test proposed by Balloux et al. (2004) revealed low values of mean correlation (<0.1) for *H–H* and *LD–LD* correlations. According to Balloux et al. (2004) (using simulated data and data from the Acevedo-Whitehouse et al. (2003)'s study on rehabilitating California sea lions *Zalophus californianus*), the expected correlation between multilocus heterozygosity and pedigree *f* would be roughly zero for such low mean correlation values. Hence the Balloux et al. (2004) test does not support the general effect hypothesis as the main underlying mechanism of the HFC observed in our population, although power may be low with 22 loci. Our findings concerning markers in genes are consistent with interesting results reported by Thelen and Allendorf (2001) who found an HFC with 10 allozymes (markers of genes or of gene rich regions), but no HFC with 10 microsatellites distributed across the genome (see also Luikart et al. 2008 and Acevedo-Whitehouse et al. 2005). We suggest therefore that the HFC we observed is mostly induced by linkage (gametic) disequilibrium between selected parts of the genome and the neutral markers used. Nonetheless, the statistical power for detecting HFCs may be low when using only 22 loci (Balloux et al. 2004). In conclusion, a combination of mechanisms ("general" effect and "local" effect) cannot be excluded; especially when we consider the relatively small number of microsatellites we used.

This study suggests that managers of wild populations of deer should carefully consider genetic variation of individuals and populations, given that genetic diversity can affect juvenile survival; indeed an HFC has been observed here with neutral markers and confirmed by the direct analysis of candidate adaptive parts of the genome (i.e., correlation with markers in important functional genes IGF1 and NRAMP). In addition, d^2 appears to be more informative than *H* in the particular conditions of our study. Theoretical studies suggest that d^2 should be more informative than *H* only in rare cases (Tsitrone et al. 2001; Goudet and Keller 2002); the results of our empirical study suggest that in the wild, situations in which d^2 measures could allow detection of HFC could be more frequent than previously expected, especially in populations with recent admixture, long-distance immigrants, and highly sedentary or philopatric animals where a strong geographic structure occurs.

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