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Short communication

Characteristics of loci and individuals are associated with germline microsatellite mutation rates in lesser kestrels (*Falco naumanni*)

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ABSTRACT

Although microsatellites are one of the most popular tools in genetic studies, their mutational dynamics and evolution remain unclear. Here, we apply extensive pedigree genotyping to identify and analyze the patterns and factors associated with *de novo* germline mutations across nine microsatellite loci in a wild population of lesser kestrels (*Falco naumanni*). A total of 10 germline mutations events were unambiguously identified in four loci, yielding an average mutation rate of 2.96×10^{-3}. Across loci, mutation rate was positively correlated with locus variability and average allele size. Mutations were primarily compatible with a stepwise mutation model, although not exclusively involved single-step changes. Unexpectedly, we found an excess of maternally transmitted mutations (male-to-female ratio of 0.1). One of the analyzed loci (Fn2.14) resulted hypermutable (mutation rate = 0.87%). This locus showed a size-dependent mutation bias, with longer alleles displaying deletions or additions of a small number of repeat than shorter alleles. Mutation probability at Fn2.14 was higher for females and increased with parental (maternal) age but was not associated with individual physical condition, multilocus heterozygosity, allele length or allele span. Overall, our results do not support the male-biased mutation rate described in other organisms and suggest that mutation dynamics at microsatellite loci are a complex process which requires further research.
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1. Introduction

Microsatellites are simple sequence nucleotide repeats characterized by their ubiquitous presence in eukaryotic genomes and extensive polymorphism [1]. Their relative abundance and high variability make them attractive genetic markers that have been increasingly used to address numerous consequential questions in both ecology and evolution [1]. Their high polymorphism (extreme in some cases; [2,3]) is in part the result of mutation rates several orders of magnitude higher than other loci [4]. This high mutability makes possible direct observations of mutation events that may be difficult to study in other DNA regions, thus opening the possibility to study and understand the factors shaping genome-wide mutation patterns [4]. Although the mechanism promoting microsatellite mutations and length variation is suspected to be the slipped-strand mispairing described by the stepwise mutation model (SMM; [5]), the factors implicated in microsatellite evolution are complex and cannot still be considered fully understood [6]. The study of microsatellite mutations is relevant not only from a theoretical point of view but also from an applied perspective as several conclusions derived from microsatellite data are often based on assumptions that are not always supported by studies on their mutation patterns [7,8].

Although patterns of microsatellite mutation have been extensively studied in humans and certain laboratory species [9–14], data on non-model organisms are less frequent [2,8,15–19]. Here, we analyze mutation patterns and rates and their potential causes for nine microsatellite loci in a wild population of lesser kestrels (*Falco naumanni*), including information for one of the most variable locus hitherto described in birds [3]. For this purpose, we applied extensive pedigree genotyping which allowed us to identify *de novo* mutations in offspring and assign them to the paternal or maternal germlines.

2. Materials and methods

2.1. Family material

Samples from lesser kestrel families were collected from a natural population located in La Mancha, central Spain, during 2001–2007 breeding seasons [20]. Monitoring included the capture and banding of breeding adults, recording of breeding

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Table 1
Characterization of microsatellite loci used for analysing germline mutations in lesser kestrels based on 333 adult (parents) individuals. Table shows number of alleles (K), expected heterozygosity (H_E), observed heterozygosity (H_O), and the probability of deviation from Hardy–Weinberg equilibrium (HWE) at each locus. HWE probabilities that remained significant following sequential Bonferroni correction for multiple tests with $\alpha = 0.05$ are shown in bold face type.

Locus	Size range (bp)	K	H_E	H_O	HWE	Repeat motif	Reference
Fn2.14	186–982	154	0.984	0.783	**<0.001**	$((AGAT)_xT)_y$	[3]
Fn1.11	258–382	17	0.763	0.765	0.928	$(AG)_{15}$ $((TG)_xTC)_y$	[3]
FV2	226–434	53	0.954	0.796	**<0.001**	–	J.H. Wetton, unpublished
FV1	120–164	12	0.508	0.539	0.256	–	J.H. Wetton, unpublished
Fp89	117–123	4	0.505	0.517	0.668	$(AT)_{12}$	[21]
Fp5	99–109	6	0.631	0.616	0.566	$(GT)_{11}$	[21]
Fp13	85–105	4	0.642	0.585	0.033	$(CA)_{12}$	[21]
Fp31	126–144	8	0.675	0.701	0.326	$(CA)_{17}$	[21]
Fp46.1	115–139	11	0.593	0.592	0.957	$(CA)_{11}$	[21]

parameters, and intensive ringing of nestlings in the colonies [20]. Blood samples (100 μl) for genetic analyses were obtained by venipuncture of the brachial vein of chicks and adults and preserved in ~1200 μl ethanol 96% at −20 °C. For the purpose of this study, we selected 651 offspring from 243 broods. In all these broods, blood samples from both parents were also available for genetic analyses.

2.2. Microsatellite genotyping

We genotyped lesser kestrel families across eight microsatellite dinucleotide loci (Fp5, Fp13, Fp31, Fp46.1, Fp89 [21]; Fu1, Fu2, J.H. Wetton, unpublished; Fn1.11 [3]) and one tetranucleotide (Fn2.14 [3]). Microsatellite characteristics and variability are described in Table 1. We used QIAamp DNA Blood Mini Kits (QIAGEN) to extract and purify genomic DNA from the blood samples. Approximately 5 ng of template DNA was amplified in 10-μl reaction volumes containing 1× reaction buffer (67 mM Tris–HCl, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, EcoStart Reaction Buffer, Ecogen), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, HEX or NED) and 0.1 U of Taq DNA EcoStart Polymerase (Ecogen). All reactions were carried out on a Mastercycler Epgradient S (Eppendorf) thermal cycler. The PCR programme used was 9 min denaturing at 95 °C followed by 30 cycles of 30 s at 94 °C, 45 s at the annealing temperature [3] and 45 s at 72 °C, ending with a 5 min final elongation stage at 72 °C. Amplification products were electrophoresed using an ABI 310 Genetic Analyzer (Applied Biosystems) and genotypes were scored using GeneMapper 3.7 (Applied Biosystems). Locus Fn2.14 presented a small proportion (2.88%) of alleles longer than 630 bp (Table 1). These particularly long alleles fall out the range detected with the size standard GS500 (Applied Biosystems) used in our usual fragment genotyping analyses. To detect these alleles we re-analyzed all homozygous individuals for locus Fn2.14 (i.e. potentially carrying a long allele not detected with the GS500 standard) using the size standard 2500-ROX (Applied Biosystems) which detect fragments up to 2500 bp long. Although the sizing accuracy of this standard is not very high (own data, unpublished), it should be noted that we have not detected any mutation involving these alleles probably due to their very low frequency in the population and because one or more members of the families carrying them were homozygous at this locus and they were not further considered for mutation analyses (see Section 2.4).

2.3. Individual characteristics

Adult birds can be unequivocally sexed in the field due to their marked sexual plumage dichromatism. Age data was readily available as several adult birds had been ringed as nestlings in the study area in previous years or captured during their first breeding years. Apart from sex and age we also determined other individual characteristics that could be associated with mutation rates [17]. These included (1) pectoral thickness, a reliable estimator of body condition in birds [22]; it was measured using a portable ultrasonic meter (Krautkrämer USM22F, Hürth, Germany, accuracy 0.1) and (2) we used two metrics to estimate individual genetic diversity: (i) homozygosity by loci (HL), a microsatellite derived measure that improves heterozygosity estimates in open populations by weighting the contribution of each locus to the homozygosity value depending on their allelic variability [23]. HL is calculated as follows: $HL = (\Sigma E_h)/(\Sigma E_h + \Sigma E_j)$, where E_h and E_j are the expected heterozygosities of the loci that an individual bears in homozygosis (h) and in heterozygosis (j), respectively [23]; (ii) uncorrected heterozygosity (H_O), calculated as the proportion of loci at which an individual is heterozygous. H_O and HL were calculated using CERNICALIN, an excel spreadsheet available on request.

2.4. Mutation detection

Whenever a suspected mutation was detected, we re-amplified and scored both parents and offspring at the mutating marker to reduce the impact of genotyping errors [10]. We assumed that the mutation was derived from the parental allele that required the least changes in repeat units. This assumption cannot be formally proven but it is a commonly applied and reasonable criterion [2,4,16]. Although lesser kestrels are mainly monogamous and extra-pair fertilizations are rare [24], non-congruence between social parent and offspring genotypes may either be due to a case of extra-pair paternity or consequence of a germline mutation [2]. To distinguish between these two possibilities we considered a case of extra-pair fertilization when a chick genotype was not congruent with one of the parent genotypes in more than two loci [2]. Some mismatches between biological parents and offspring alleles can also result from the transmission of null alleles (non-amplifying alleles) or when certain alleles do not amplify for other reasons (allelic drop-outs) [4,8,16]. This is likely to be an important problem for three loci that deviated from Hardy–Weinberg equilibrium probabilities (Table 1). For these reasons, we only considered meiosis derived from families where both parents and the offspring were all heterozygous for two amplifying alleles [8,16]. With this very conservative criterion we excluded the possibility that a transmitted non-amplified allele in an apparently homozygous parent is interpreted as a germline mutation. Although this criterion is expected to reduce the absolute number of mutations detected, there is no reason to think that it could underestimate mutation rates.

3. Results and discussion

3.1. Rate and patterns of microsatellite mutations

We have typed nine microsatellite loci in 651 offspring, representing 11718 meioses. Of these, 3374 meioses derived from families where both parents and the offspring were all heterozygous for two amplifying alleles. Hereafter, all data and analyses are only referred to these 3370 meioses unambiguously assigned to paternal or maternal germlines. As a result of this approach, the number of male and female meioses considered is equal. It should be noted that after applying such a conservative criterion the number of meiosis analyzed varied between loci because the probability of heterozygosity is greatly dependent on locus variability (Table 1). Thus, the number of meioses considered for each of the nine analyzed loci is Fn2.14 = 576; Fn1.11 = 588; FV2 = 518; FV1 = 236; Fp89 = 148; Fp5 = 352; Fp13 = 234; Fp31 = 362; Fp46.1 = 360.

We have identified 10 independent *de novo* mutations (Table 2). The overall average mutation rate of the nine loci was 2.96×10^{-3}.

Table 2
Germline mutations detected at four microsatellite loci in lesser kestrel families. Only unambiguous mutant alleles (i.e. detected when both parents and offspring appear heterozygous for the amplifying locus) have been included. The mutant allele in offspring and the most likely progenitor mutant allele are indicated by bold face type.

Locus	Parental genotypes		Offspring genotype	Size change (bp)
	Paternal	Maternal		
Fn2.14	230–190	**290**–266	**302**–230	+12
Fn2.14	494–208	654–**308**	494–**316**	+8
Fn2.14	**254**–190	578–274	578–**258**	+4
Fn2.14	352–200	**348**–210	352–**344**	−4
Fn2.14	430–246	310–**238**	246–**242**	+4
FV2	312–298	300–**276**	298–**274**	−2
FV2	308–228	**294**–226	**298**–228	+4
FV2	318–252	296–**288**	**282**–252	−6
Fp5	107–105	109–**105**	107–**103**	−2
Fp46.1	127–125	**129**–119	**131**–125	+2

The mutation rate for the four loci where mutant alleles were detected was 5.54×10^{-3} (8.68×10^{-3} for Fn.2.14, 5.80×10^{-3} for FV2, 2.84×10^{-3} for Fp5, and 2.78×10^{-3} for Fp46.1). These values are within the range reported for microsatellite loci in other organisms [4], including birds [17,18]. We analyzed whether mutation rate was associated with locus variability estimated by the total number of alleles (K) observed in the 333 parents analyzed (thus, standardized for sample size) and expected heterozygosity (H_E) using simple linear regressions. The precision of mutation rates could be different because sample size (i.e. the number of meioses analyzed; see above) used for their estimation varied among loci. So, we used sample size to give observations different weights in a weighted least-square (WLS) analysis. This analysis revealed that mutation rates were positively correlated with both allelic diversity ($r = 0.827$; $n = 9$; $t = 3.892$; $P = 0.006$) and expected heterozygosity ($r = 0.762$; $n = 9$; $t = 3.115$; $P = 0.017$). Further, mutation rates were positively associated with average number of repeats at a locus (WLS simple linear regression: $r = 0.676$; $n = 9$; $t = 2.427$; $P = 0.046$) and average allele length (WLS simple linear regression: $r = 0.763$; $n = 9$; $t = 3.128$; $P = 0.017$). All these correlations remained significant after applying a sequential Bonferroni correction (with $\alpha = 0.05$) for multiple probability estimation [25]. This agrees with previous research suggesting that long loci mutate more often than short loci both within [13] and across species [26]. This could occur if the likelihood of replication slippage increases in a repetitive sequence with many repeat numbers [4]. Another possible explanation is that young loci particularly unstable for unknown reasons have initially evolved from shorter alleles up they have reached an equilibrium at longer allele lengths than loci showing intrinsically lower mutation rates, i.e. higher mutability in certain loci is responsible of larger allele length rather than *vice versa*. It should be noted that all the variables analyzed (i.e. K, H_E, average number of repeats, and average allele length) are highly inter-correlated among them (Pearson's correlations: $P < 0.05$ in all cases), and so the correlations observed are all the result of an ultimate association between mutation rate and different parameters related with locus variability.

All observed mutations resulted in alleles whose sizes were identical to alleles already present in the study population. As found in several other studies, this result suggests that size homoplasy is a widespread phenomenon [7,27]. We did not find clustered mutations, which may in part be consequence of the small size of the studied families [7,8,17]. Surprisingly, there was an excess of maternally derived mutations over the four loci where mutant alleles were detected (binomial test (two tailed): $P = 0.021$; male-to-female ratio of 0.1). One mutation arose from a progenitor allele on the paternal side and nine on the maternal size and no mutation was ambiguous with respect to maternal *vs*. paternal origin (Table 2). This is opposite to previous studies suggesting that germline mutations are more frequent in males than in females [10,15,28], a pattern that would be expected if the number of germline cell divisions is higher in males and mutations are predominantly replication-dependent. However, male-biased mutation rates are not a general rule and several studies have also found an opposite pattern (birds: [2,8,18]; reptilian: [19]; insects: [29]) or even a locus-specific pattern within species (humans: [10,30]; *Hirundo rustica*: [2,8,15]; *Malurus cyaneus*: [18]). We explored other potential factors that may have influenced the observed female-biased mutation rate [19]. Larger alleles have been found to mutate more frequently than smaller ones [10,19] and large size differences between the two microsatellite alleles in an individual (i.e. allele span) can also result in an increased mutation rate [12]. However, the mutant allele was neither the largest transmitted one (paired t-test: $t = -1.365$, $P = 0.205$) nor that originating from the parental side with higher allele span (paired t-test: $t = -1.395$, $P = 0.197$).

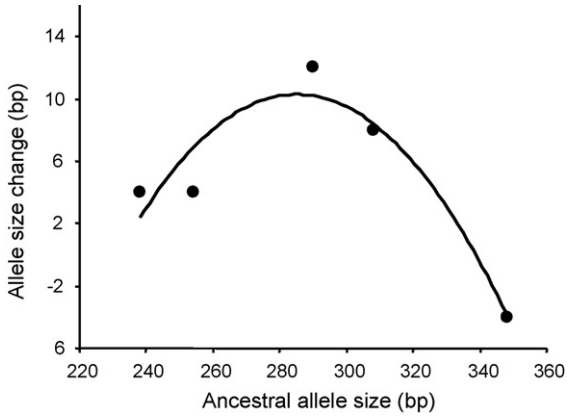

Fig. 1. Magnitude and direction (bp, allele size change) of germline mutations at Fn2.14 microsatellite locus in relation to the size of the progenitor allele (bp, ancestral allele size). The quadratic regression line is shown.

Therefore, the observed bias towards maternal mutations cannot be explained by these factors.

Most mutant alleles differed from the parental allele by an increase or decrease of just one repeat unit (Fn2.14: 3/5; FV2: 1/3; Fp5: 1/1; Fp46.1: 1/1; Table 2). This pattern agrees with previous studies that have found that most mutations involve single repeat changes consistent with a step-wise model of microsatellite evolution [10,17]. Four mutations involved more than one repeat unit: at Fn2.14 two mutations added two and three repeats, whereas at FV2 a mutation removed three repeats and another added two repeats (Table 2). Expansions ($n = 6$) were more frequent than contractions ($n = 4$) over the four loci where mutant alleles were detected, but this was far of being significantly different from equity (binomial test (two tailed): $P = 0.754$). Only considering the locus Fn.2.14 (i.e. the locus with the higher number of mutations detected; Table 2) we found an effect of ancestral allele size on allele size change: a multiple regression analysis showed that allele size change increased (linear term: $\chi^2 = 13.70$; $P = 0.002$) and then declined (squared term: $\chi^2 = 13.95$; $P = 0.002$) with ancestral allele size (Fig. 1). Although sample sizes are very low, our data suggest that longer alleles are more likely to display deletions or additions of a small number of repeats than shorter alleles, a pattern that may prevent infinite growth (i.e. an "upper length ceiling") and explain the stationary allele distribution generally observed in most microsatellites [10,14]. In any case, it should be noted that large allele drop-out in this locus may have biased the size distribution of mutating alleles.

3.2. Factors influencing mutation probability at Fn2.14 locus

The locus Fn2.14 is one of the most variables loci hitherto described in birds and it showed during the genotyping process mutation rates (0.87%) typical of previously described hypermutable loci [2,8,15]. Although comparisons across the analyzed loci provided evidence that mutation rate increases with average allele length, allele size was not associated with the probability of mutation within Fn2.14 locus (logistic regression: $\chi^2 = 0.18$; $n = 576$; $P = 0.668$). After this consideration, we analyzed the characteristics of individuals potentially associated with probability of germline mutations in locus Fn2.14. For this purpose we used a Generalized Linear Mixed Model (GLMM) implemented with the GLIMIX macro of SAS [31]. Probability of mutation for a given meiosis ($n = 576$) was analyzed using a binomial error structure and logit link. The identity of colony, parent identity and cohort, brood identity (nested within colony identity), and breeding year were included as ran-

Table 3
GLMM (binomial error and logit link function) for probability of germline mutation at Fn2.14 microsatellite locus. Parameter estimates ± S.E. for the levels of fixed factor was calculated considering a reference value of zero for the "female" level in the variable "sex".

	Estimate ± S.E.	Test	*P*
Explanatory terms			
Intercept	−23.553 ± 2.260		
Sex	3.276 ± 1.028	$F_{1,573} = 10.16$	0.002
Parental age	3.335 ± 0.474	$F_{1,573} = 49.46$	<0.001
Rejected terms			
Parental pectoral thickness		$F_{1,572} = 3.07$	0.080
Homozygosity by loci (*HL*)		$F_{1,572} = 0.01$	0.985
Uncorrected homozygosity (H_O)		$F_{1,572} = 0.01$	0.983
Allele span		$F_{1,572} = 0.51$	0.474
Covariance parameter estimates			
Colony identity	0	–	–
Parental identity	0	–	–
Parental cohort	4.211 ± 3.578	$Z = 1.18$	0.120
Brood identity	0	–	–
Breeding year	0	–	–
Allele identity	17.178 ± 4.808	$Z = 3.57$	<0.001

dom effects in the manner of a randomized complete block design to avoid pseudoreplication [32]. Further, we included allele identity as an additional random effect to control for potential differences in mutation rates among alleles [17]. Mutation probability was higher in females (Table 3), suggesting that some particular characteristics of oogenesis could have resulted in female-biased mutation rates. Further, mutation probability was positively associated with individual age (Table 3; Fig. 2). All other analyzed variables, including physical condition (pectoral thickness), individual genetic diversity (estimated as *HL* and H_O), and allele span (i.e. the difference in length between alleles of a heterozygote; [12]) were not associated with mutation probability (Table 3). Accordingly, previous studies on avian hypermutable microsatellite loci have neither found any association between mutation probability and physical condition/heterozygosity [17] whereas the correlations with allele span are similarly lacking [17,18] or difficult to disentangle from the effects of allele length [15]. The association with age is particularly intriguing, because most detected mutations are from the female germline. In fact, the relationship between probability of mutation and age was still highly significant when the analysis was restricted to females ($F_{1,286} = 15.63$; $P < 0.001$). Although the number of detected mutations was fairly low this is, to the best of our knowledge, the fist study finding an association between probability of germline mutations and maternal age at a microsatellite locus. The only previous study analyzing the association between age and mutation probability in birds did not find any effect, although it was only explored in males [17]. In humans, *de novo* germline mutations have been also found associated with advanced maternal age [33], suggesting that the pattern observed for Fn2.14 locus is not unique. Overall, our results do not support the male-biased mutation rate described in other organisms and suggest that mutation dynamics at microsatellite loci are a complex process which requires further research.

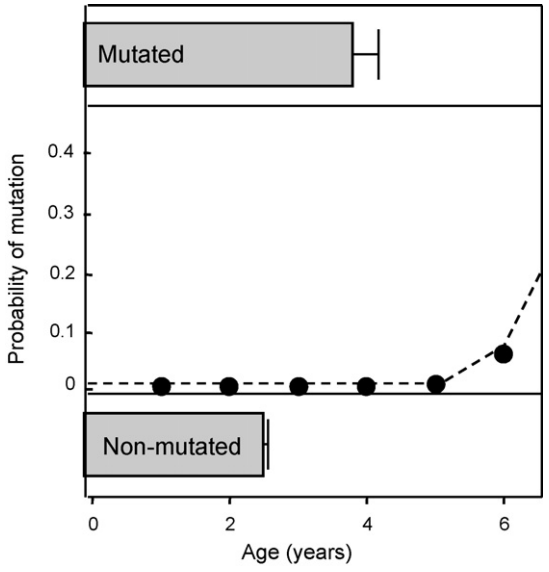

Fig. 2. Probability of mutation at locus Fn2.14 as a function of parental age. Model predictions have been generated for the range of ages covering the variability observed in the study population. Shaded bars indicate actual mean ± 1 S.E. age of individuals transmitting mutated/non-mutated alleles.

Conflict of interest

We declare that there are no conflicts of interest.

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