MLH1-focus mapping in birds shows equal recombination between sexes and diversity of crossover patterns

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Abstract

Using immunolocalization of the mismatch-repair protein MLH1 in oocytes and spermatocytes of the Japanese quail and the zebra finch, we estimated the average amount of recombination in each sex of both species. In each case the number of MLH1 foci is statistically equivalent in males and females and the resulting sex-averaged map lengths are 2800 cM in the Japanese quail and 2275 cM in the zebra finch. In the Japanese quail the MLH1 foci are regularly distributed along the macrobivalents and recombination rates per Mb pair are somewhat lower compared to the chicken. In the zebra finch the MLH1 foci on the macrobivalents are substantially reduced in number relative to the Japanese quail and they show remarkable localization in both sexes. It is proposed that the lack of sex-dependent differences in recombination might be an extended feature among birds and that the different recombination patterns observed here reflect different controls of crossing over in spite of similarities regarding karyotypic asymmetry and DNA content. We discussed possible causes of the differences between birds and mammals, which show sex-dependent recombination differences.

Introduction

Cytological analyses of the crossing over in the chicken and the pigeon showed that recombination rates are similar in both sexes in each species. In the chicken the initial evidence was obtained comparing the number of recombination nodules observed by electron microscopy in oocytes during pachytene (Rahn & Solari 1986) to the number of chiasmata reported in spermatocytes at diakinesis (Pollock & Fechheimer 1978). Data from chiasma counts in lampbrush bivalents of chicken oocytes and from the linkage maps also demonstrated equal recombination in both sexes of this species (Rodionov *et al.* 1992, reviewed in Schmid *et al.* 2000). In the pigeon the lack of sex differences in crossing over was shown by recombination nodule mapping in synaptonemal complex (SC) spreads of oocytes and spermatocytes (Pigozzi & Solari 1999a,b). These results led to the assumption that sex-dependent differences in crossing over are absent in birds.

When comparing crossover data between sexes it is important to use the same methodology since map lengths estimated from linkage analysis often show discrepancies with estimates using cytological markers in the same species. In most birds linkage data are not available or the number of informative markers is insufficient to compare recombination throughout the whole genome in both sexes. An alternative approach to crossing over is to analyze the distribution of the mismatch-repair protein MLH1, which localizes on discrete foci along the synaptonemal complexes

during pachytene. MLH1 foci have been shown to correspond in number and distribution to chiasmata in mice and humans (Baker *et al.* 1996, Barlow & Hultén 1998, Anderson *et al.* 1999, Froenicke *et al.* 2002) and they are the counterparts of recombination (late) nodules observed with electron microscopy. Since the structure of the MLH1 protein is evolutionarily conserved, an antibody raised against MLH1 protein of mammalian origin also recognizes the homologous protein in birds, as shown by the immunolabeling of MLH1 foci along the SCs of chicken and other avian species (Pigozzi 2001, Pigozzi & Solari 2005, M.I. Pigozzi, unpublished).

In the present study the frequencies and patterns of MLH1 foci are compared in males and females of two evolutionarily distant species: the Japanese quail (Galliformes) and the zebra finch (Passeriformes) in order to test the conservation of equal recombination between sexes. This is the first report that compares MLH1 foci in both sexes in birds and the only data using this methodology besides the comparison of MLH1 foci in human oocytes and spermatocytes (Tease & Hultén 2004). The selected species have typical asymmetric karyotypes, with few macrochromosomes and numerous microchromosomes, and therefore possible crossover variations between species would not respond to different proportions of DNA distributed in macro vs. microchromosomes or to large variations in the number of microbivalents.

Sex-dependent recombination differences seem to be the rule among mammals, as shown by comparisons of cytological and/or molecular crossover maps in both sexes in mice and humans (Dietrich *et al.* 1996, Broman *et al.* 1998, Tease *et al.* 2002). If the lack of recombination differences in males and females is generalized in birds, then they could serve as a test group to look for the causes of the mentioned differences in mammals.

Materials and methods

Meiotic cells for synaptonemal complex spreads of the Japanese quail were obtained from ovaries of females 1–3 days after hatching and from the testes of adult males. SC spreads of oocytes and spermatocytes of the zebra finch were obtained from females 4–5 days old and from adult males. Meiotic spreads and immunodetection were done employing previously described methods (Pigozzi 2001). Primary

antibodies were: rabbit anti-SCP3 (P. Moens, York University, Canada) or anti-SMC3, that label the lateral elements of the synaptonemal complex, anti-MLH1 of mouse origin (BD, Pharmingen) and CREST serum that recognizes centromeric proteins (W. Brinkley, Baylor College of Medicine, USA). Suitable combinations of secondary antibodies (Jackson ImmunoResearch) were used to label the synaptonemal complexes and the centromeres in red and the MLH1 foci in green. Separate images for each color were captured using a CCD digital camera (Leica Microsystems) and merged using Adobe Photoshop. Measurements were done using the program Micromeasure (Reeves 2001), that registers the number and position of MLH1 foci along the synaptonemal complexes, and statistical tests and graphs were done with GraphPad Prism 4.0.

Results

The SC karyotype in the Japanese quail

In spreads from pachytene nuclei the complete set of synaptonemal complexes can be observed in oocytes and spermatocytes showing the 38 autosomal bivalents and the sex pair (Figure 1a and b). There is very good agreement regarding length and centromeric index of the SCs compared to the same parameters from mitotic macrochromosomes (Stock & Bunch 1982, Shibusawa *et al.* 2001). The longest SC is submetacentric, the second is nearly metacentric and SCs 3–7 have either terminal or near-terminal centromeres. The ZW pair forms an asymmetric bivalent with the longest axis corresponding to the Z and the shorter one corresponding to the W, while the ZZ bivalent is almost metacentric and the fifth in size. Thus, the largest macrobivalents and the sex bivalent can be individualized after SC measurements and centromere identification as shown in Figure 1. The centromere labeling with CREST revealed that most microchromosomes are metacentric (Figure 1), a very different feature from chickens–closely related to quails–where electron microscopy showed that all microbivalents have near-terminal kinetochores (Solari 1977, Rahn & Solari 1986). The observations after immunostaining were confirmed by re-examination of quail pachytene oocytes stained with phosphotungstic acid and photographed at the

electron microscope (A.J. Solari, personal communication). In Figure 1a, SCs and centromeres appear red as observed in the nuclei used for MLH1 analysis (see below). Additionally, two-color immunostaining with CREST and SCP3 was used on a sample of spermatocytes to confirm the number of microchromosomes with median and terminal centromeres (Figure 1b). There are five microchromosomes that are acrocentric (indicated by arrows in Figure 1) and 26 that are metacentric; the acrocentric are among the largest microbivalents, with the exception of one that is a medium-sized microchromosome.

MLH1 foci in the Japanese quail

A total of 81 well-spread spermatocytes and 60 oocytes showing complete SC sets were analyzed to score the number of MLH1 foci and centromere positions in this species (Figure 2a and b). Since the sex pairs ZZ and ZW have different morphological features and show unrestricted vs. restricted recombination respectively, only data from the autosomal bivalents were used to compare recombination frequencies and SC lengths between sexes. The average number of foci in the largest bivalents and the total number of foci in the autosomal SC sets of males and females are listed in Table 1 along with comparative data in chicken oocytes. Statistical comparison with a t-test showed that the average numbers of MLH1 foci in oocytes and spermatocytes are not significantly different ($p = 0.12$). Similarly, the lengths of the complete autosomal SC set in males (231.2 µm ± 28.6) and females (238.8 µm ± 33.5) do not differ significantly ($p = 0.30$). In both sexes the MLH1 foci can be found along any segment of the SC without obvious localization (Figure 2). Most microbivalents had one focus, with the exception of the largest microbivalent–the eighth in size–that had two foci in one-third of the nuclei, and microbivalents without foci were rare (less than 1%).

MLH1 foci in the zebra finch

In spreads of zebra finch oocytes and spermatocytes there are 39 autosomal SCs, the sex pair and a germline-restricted chromosome that forms a bivalent in oocytes but is a univalent with condensed chromatin in spermatocytes. The sex-dependent behavior of the germline-restricted chromosome was described else-
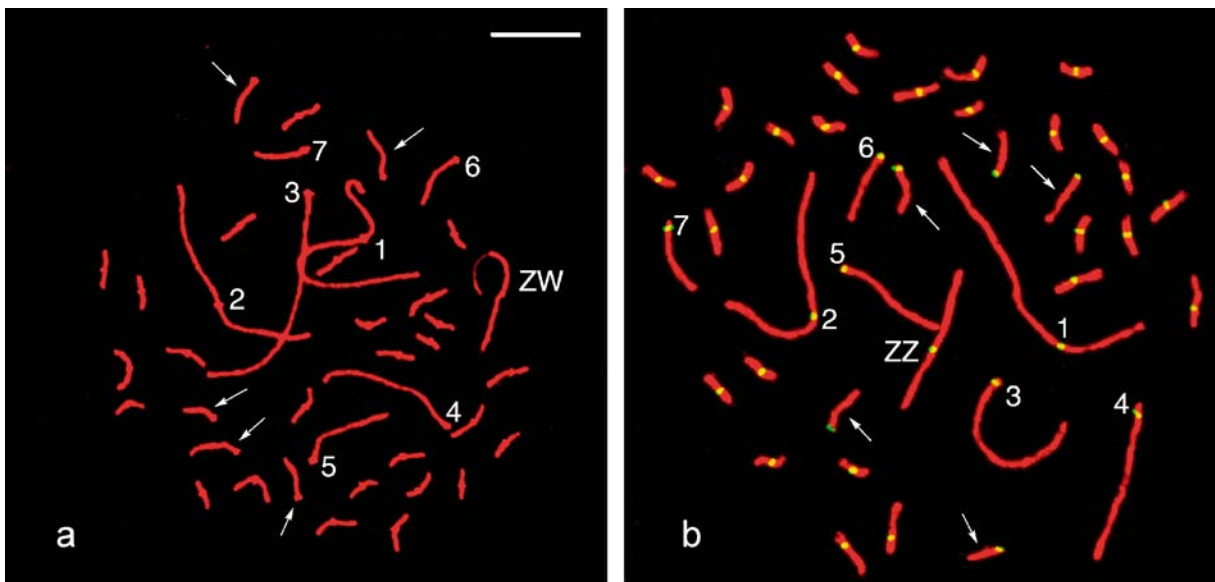

Figure 1. Synaptonemal complex spreads from the Japanese quail immunostained with anti-SCP3 and CREST serum. (**a**) Pachytene oocyte showing the complete set of autosomal synaptonemal complexes and the sex pair (ZW). Centromeres are seen as red dots bulging from the linear SC. The numbers next to the centromeres indicate the macrobivalents SCs. (**b**) Spermatocyte in pachytene with the centromeres marked in yellow and the synaptonemal complexes in red. The arrows point at the five microbivalents that are acrocentric. Bar: 10 µm.
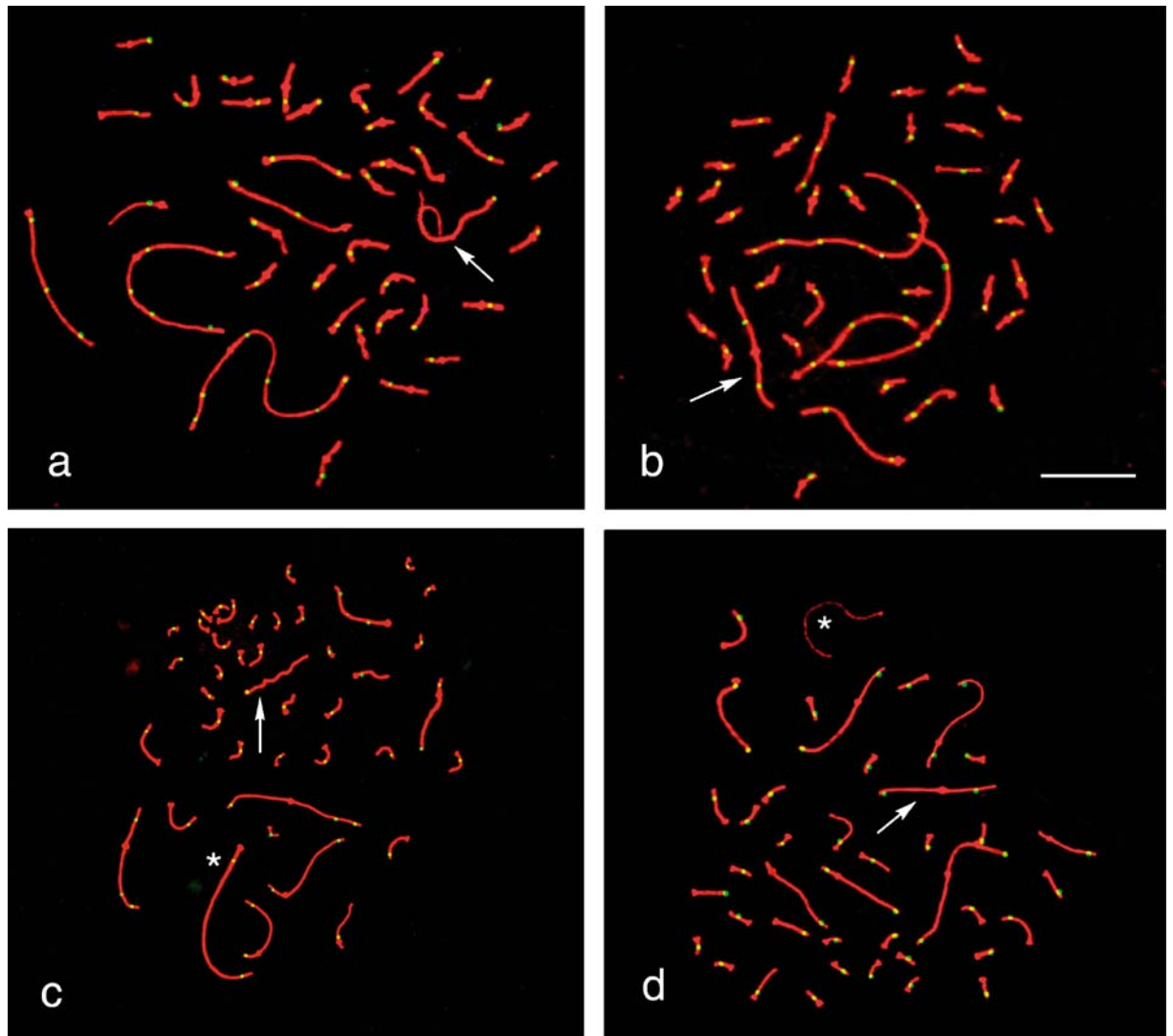
Figure 2. MLH1 foci in the Japanese quail (**a**, **b**) and the zebra finch (**c**, **d**). The MLH1 foci appear as yellow dots along the synaptonemal complexes (red). Note the higher number of foci in the macrobivalents of the Japanese quail and the longer SCs compared to the zebra finch. In the zebra finch the MLH1 foci show a trend to locate on extreme positions of the synaptonemal complexes both in oocytes and spermatocytes. The arrows point at the sex bivalent, ZZ or ZW and the asterisks mark the germline-restricted chromosome. Bar: 10 µm.

where (Pigozzi & Solari 2005) and the correspondence between the largest SCs and mitotic chromosomes was also established in a previous report (Pigozzi & Solari 1998). Therefore, similarly to the quail, in the zebra finch it is also possible to count the MLH1 foci identifying individual macrobivalents by its relative length and its centromeric index. In a sample of 41 oocytes and 59 spermatocytes the average length of the autosomal SC set in the female (154 µm ± 25) was slightly longer than in the male

(141 µm ± 8.7). These values are statistically significantly different ($p = 0.002$), although the difference might be a consequence of the selection of the spermatocytes used for MLH1 focus analysis. Spermatocytes with shorter SC sets showed stronger labeling of MLH1 foci, instead no differences in MLH1-focus intensity were observed among oocytes with longer or shorter SCs. Therefore the shorter SC length observed in spermatocytes compared to oocytes can be ascribed to the sampling procedure

Table 1. Average numbers of MLH1 foci and standard deviations in the autosomal bivalents of quail oocytes and spermatocytes compared to the chicken

Quail			Chicken*
Bivalent	Spermatocytes	Oocytes	Oocytes
1	6.1 ± 0.8	6.6 ± 0.9	9.0 ± 1.4
2	4.8 ± 0.8	5.1 ± 0.7	6.9 ± 1.2
3	3.7 ± 0.8	4.0 ± 0.9	4.9 ± 0.9
4	3.2 ± 0.5	3.0 ± 0.7	4.1 ± 0.6
5	2.1 ± 0.2	2.1 ± 0.3	3.3 ± 0.6
6	1.4 ± 0.5	1.6 ± 0.5	1.9 ± 0.5
7	1.5 ± 0.5	1.4 ± 0.5	2.1 ± 0.5
Total	56.3 ± 1.8	55.3 ± 2.1	65 ± 4.0

*Data from Pigozzi 2001.

and probably does not represent a real difference between sexes. The number of MLH1 foci in oocytes and spermatocytes (Figure 2c and d) is noticeably lower than those observed in the Japanese quail, in correlation to shorter SC lengths. In the same sample used for SC measurements the average number of foci in the autosomal set was 45.7 ± 0.4 and 45.2 ± 0.2 in oocytes and spermatocytes, respectively and a *t*-test showed that these values are not significantly different ($p = 0.18$). The largest SC never shows more than three foci, and the most frequent number per macrobivalent is two (Table 2). Both in oocytes and spermatocytes there is a trend of MLH1 foci to localize towards the distal regions of each arm in metacentric or submetacentric bivalents, while in the acrocentrics such as bivalents 6 or 7, foci group toward the centromere and the distal region of the arm. This distribution leaves a relatively large stretch with low frequency of foci in all macrobivalents

Table 2. Average numbers of MLH1 foci and standard deviations in the autosomal bivalents of zebra finch oocytes and spermatocytes

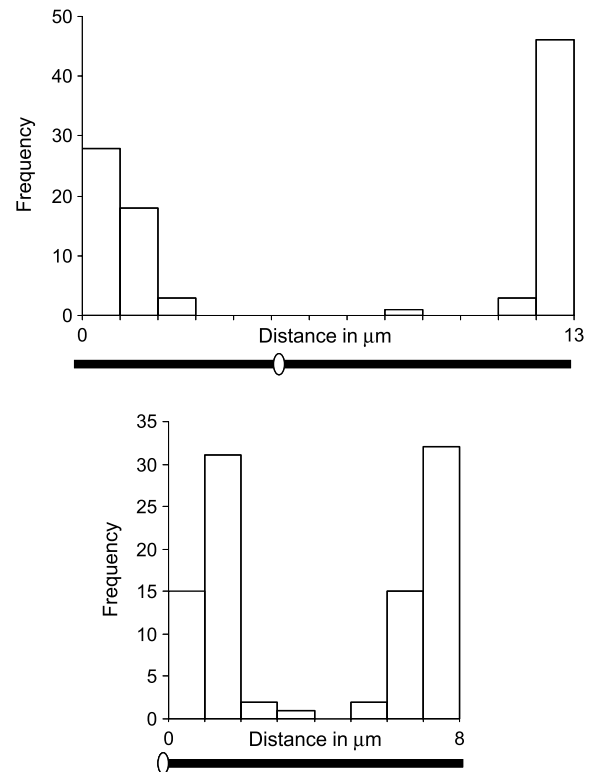| Bivalent	Spermatocytes	Oocytes
1	2.1 ± 0.4	2.3 ± 0.5
2	2.1 ± 0.2	2.2 ± 0.4
3	2.0 ± 0.2	2.0 ± 0.5
4	1.9 ± 0.4	1.9 ± 0.3
5	1.9 ± 0.4	2.0 ± 0.4
6	1.9 ± 0.3	2.0 ± 0.3
7	1.4 ± 0.5	1.5 ± 0.5
Total	45.2 ± 0.2	45.7 ± 0.4

Figure 3. Histograms showing the localization of the MLH1 foci in the zebra finch. The *x* axis represents the average SC length in micrometers and the height of the bars indicates the cumulative number of foci in each 1-µm interval in a sample of 59 spermatocytes. Top: frequency histogram of MLH1 foci in the submetacentric chromosome 2. Bottom: the same representation in the acrocentric bivalent 6. Below each graph a diagram shows the location of the centromere (white oval) on each synaptonemal complex (black line).

(Figure 2c and d). This region with low number of foci includes the centromere in biarmed bivalents and it is in the middle of the long arm in acrocentric macrobivalents. Figure 3 depicts these particular patterns of recombination in one biarmed and one acrocentric bivalent as frequency histograms of MLH1 foci built from spermatocyte data.

Discussion

Different crossover patterns in birds

Recombination studies suggested that crossover rates and patterns are fairly constant among birds, even though this idea may be biased by the fact that most

analyses are focused on Galliformes given the importance of poultry to the global food supply. In this trend, MLH1 focus mapping among birds has been reported only in *Gallus domesticus* (Pigozzi 2001), where the number of foci matches well with the number of recombination nodules (Rahn & Solari 1986). Our present results support the reliability of MLH1 foci to estimate recombination rates in birds, since the mean number of MLH1 foci in the four largest macrobivalents of the Japanese quail is close to the number of chiasmata reported for the corresponding lampbrush bivalents of the same species (Rodionov & Chechik 2002). On the other hand, the sex-averaged genetic map of 2800 cM calculated from the mean number of MLH1 foci is considerably lower than the 3800 cM estimated from linkage analysis (Kayang *et al.* 2004). This type of discrepancies between cytological maps and genetic maps are common in different organisms and this has been discussed in relationship to chicken data by Rodionov *et al.* (2002). The distribution of MLH1 foci on the largest SCs of the Japanese quail is similar to that in the chicken (Pigozzi 2001), i.e. with no apparent localization of crossing over events that are then more or less evenly spaced along macrobivalents. This pattern was also observed for recombination nodules on the SCs of the pigeon (Pigozzi & Solari 1999a,b) and the chiasmata on lampbrush macrobivalents from different Galliformes (Rodionov & Chechik 2002). The Japanese quail shows lower numbers of MLH1 foci compared to the chicken, mainly because of lower amounts in the largest bivalents, although microbivalents with two foci are also less frequent than in chicken oocytes. The fact that most microbivalents in the Japanese quail are metacentric does not change the pattern of one crossover in most microbivalents demonstrated in chickens and pigeons, where microbivalents are instead acrocentric (Rahn & Solari 1986, Pigozzi & Solari 1999a,b). This suggests that in small chromosomes the minimal interference distance between crossovers does not change because of the presence of the centromere, and also that there are no requirements of one crossover per chromosome arm as proposed in mammals (reviewed in Pardo-Manuel de Villena & Sapienza 2001). As far as we know this is the first report that shows that most microchromosomes in the quail are metacentric. In fact, among 17 species from nine different orders examined in our laboratory using SC spreads for electron micros-

copy or immunostaining, this is the only species with this large number of metacentric microchromosomes. Comparative gene mapping with the chicken will establish if these numerous centromere changes in the Japanese quail occurred in the absence of syntenic gene order rearrangement or if they are a consequence of multiple pericentric inversions, as occurred in some macrochromosomes in this species (Shibusawa *et al.* 2001).

In contrast to the recombination pattern observed in the other birds examined so far, the zebra finch shows localized recombination in the macrobivalents, pointing to different controls for crossover distribution in this species. This particular distribution of the recombination events is not correlated with special features regarding G-banding or replication studies of the macrochromosomes in the zebra finch because they do not depart from what is generally observed in birds (Takagi 1972, Itoh & Arnold 2005). The recombination 'valleys' observed in all macrobivalents are not related to the presence of heterochromatin, which is known to have very few or no crossovers. C banding shows that large blocks of heterochromatin are not present in the macrochromosomes of the zebra finch, with the exception of the long arm of the Z chromosome and the pericentromeric region of chromosome 6 (Christidis 1986, M.I. Pigozzi, unpublished). It is interesting to notice that the lower crossover rates in the zebra finch do not respond to DNA content variations that, in this species, are very similar to the average for most birds (Peterson *et al.* 1994) but they are accompanied by shorter SC lengths (see Results). These features are in agreement with the known relationship between SC length and recombination rates observed in a wide number of taxa (Sherman & Stack 1995, Froenicke *et al.* 2002, Lynn *et al.* 2002), and also with the fact that SC/DNA ratios are positively correlated with recombination rates (Peterson *et al.* 1994).

It is important to mention that the lower crossover rate observed in the macrobivalents of the zebra finch might not be an exceptional feature among birds. We have observed that at least one psitacid species has comparatively low numbers of MLH1 foci similarly localized as shown in the zebra finch (M.I. Pigozzi, unpublished). Also, linkage mapping in a passerine bird, although limited to loci on seven chromosomes, showed relatively low recombination rates when compared to chickens (Hansson *et al.*

2005). Altogether these data point to the existence of at least two different patterns of recombination in birds: one showing a higher number of crossovers per Mb pair and without localization, and another with lower recombination rates and localized crossing over on macrobivalents. It is possible that other crossover patterns can be revealed by additional analyses in other avian species. These variant recombination patterns may affect the levels of diversity between species and their genome composition, as well as having practical consequences for genetic mapping of chicken orthologs in other birds.

The absence of sex-dependent differences of crossovers in birds compared to their presence in mammals

Differences of crossing over frequencies have been observed in a number of organisms, and even though the most extensive investigations have been done in human meiocytes, these differences have also been observed in plants and invertebrates (see Lenormand 2003 for a review). In birds, instead, such differences seem to be absent, as shown by our present results and previous cytological and linkage map analyses in other birds, which show equal amounts of global recombination in both sexes in four species from three different orders: the chicken, the common pigeon, the Japanese quail and the zebra finch. There is a single report showing dimorphic recombination rates in males and females of a passerine bird (Hansson *et al.* 2005). In this report longer map distances between markers were observed in the female, but it would be necessary to extend these analyses to cover the whole genome to rule out the existence of loci with higher recombination in males that might compensate the longer distances observed in females.

In humans, sex-dependent differences in recombination have been related to differential SC lengths in oocytes and spermatocytes and to the formation of shorter DNA loops in oocytes than spermatocytes (Tease & Hultén 2004). Data from SC length in birds support this relationship since the average SC length in oocytes and spermatocytes from chickens (Kaelbling & Fechheimer 1983, Rahn & Solari 1986), pigeons (Pigozzi & Solari 1999a,b) and the Japanese quail (this report) are very similar, and recombination rates do not show differences between

sexes in the same species. Even the difference in SC length observed here between oocytes and spermatocytes of the zebra finch is small and might be related to sampling error. It must be pointed out that, in spite of this agreement in higher vertebrates, the cause–effect relationship of SC length and recombination frequencies has not been established, since it is possible that recombination events actually cause the differences observed in SC length by influencing the organization of meiotic chromosomes (Quevedo *et al.* 1997, Kleckner *et al.* 2003).

In mammals sex-dependent recombination patterns have been related to the different times of meiotic development in females and male germ cells (Lenormand 2003, Tease & Hultén 2004). The fact that avian oocytes and spermatocytes also start meiosis at different times of development indicates that the same assumption is not valid for birds. The actions of epigenetic factors, such as genomic imprinting, have also been related to sex differences in crossing over, since imprinted loci showed different recombination rates between sexes in mammals (Paldi *et al.* 1995). If genetic imprinting is absent in birds (see Dunzinger *et al.* 2005 and references therein), this could be related to the lack of sex differences in recombination in male and female meiosis, as opposed to mammals.

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