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Hybrid speciation in sparrows II: a role for sex chromosomes?

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Abstract

Homoploid hybrid speciation in animals is poorly understood, mainly because of the scarcity of well-documented cases. Here, we present the results of a multilocus sequence analysis on the house sparrow (*Passer domesticus*), Spanish sparrow (*P. hispaniolensis*) and their proposed hybrid descendant, the Italian sparrow (*P. italiae*). The Italian sparrow is shown to be genetically intermediate between the house sparrow and Spanish sparrow, exhibiting genealogical discordance and a mosaic pattern of alleles derived from either of the putative parental species. The average variation on the Z chromosome was significantly reduced compared with autosomal variation in the putative parental species, the house sparrow and Spanish sparrow. Additionally, divergence between the two species was elevated on the Z chromosome relative to the autosomes. This pattern of variation and divergence is consistent with reduced introgression of Z-linked genes and/or a faster-Z effect (increased rate of adaptive divergence on the Z). F_{ST}-outlier tests were consistent with the faster-Z hypothesis: two of five Z-linked loci (*CHD1Z* and *PLAA*) were identified as candidates for being subject to positive, divergent selection in the putative parental species. Interestingly, the two latter genes showed a mosaic pattern in the (hybrid) Italian sparrow; that is, the Italian sparrow was found to be fixed for Spanish sparrow alleles at *CHD1Z* and to mainly have house sparrow alleles at *PLAA*. Preliminary evidence presented in this study thus suggests that sex chromosomes may play a significant role in this case of homoploid hybrid speciation.

Keywords: birds, faster-X, homoploid hybrid speciation, multilocus analysis, Z chromosome

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Introduction

Speciation is usually portrayed as a bifurcation process in which the resulting lineages diverge over evolutionary time by natural selection and genetic drift. Associated with the divergence, the lineages accumulate genetic incompatibilities and other barriers to gene flow and eventually become reproductively isolated. This development of reproductive isolation is inarguably an important event in the evolutionary history of sexually reproducing organisms because the homogenizing effects of gene flow are brought to a final halt, allowing genetically independent evolution of the resulting lineages (Coyne & Orr 2004). However, full reproductive isolation has far from universally been reached among the recognizable phenotypic clusters of individuals we commonly refer to as species. Recent estimates suggest that about 10% of animal and 25% of plant species are involved in hybridization and introgression with at least one other species (Mallet 2005, 2007). Such hybridization can have evolutionary consequences and may even be an alternative route to the formation of new species under certain conditions: either accompanied by a change in ploidy level (polyploid hybrid speciation) or not (homoploid hybrid speciation) (Rieseberg 1997; Mallet 2005, 2007; Arnold 2006; Mavárez & Linares 2008; Nolte & Tautz 2010; Brelsford *et al.* 2011). However,

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particularly the latter mode of hybrid speciation has so far been little investigated and is poorly understood compared with bifurcating speciation (Mavárez & Linares 2008; Duenez-Guzman *et al.* 2009).

Studies on the genetic basis of reproductive isolation suggest that the sex chromosomes (X and Z in male and female heterogametic taxa, respectively) are hotspots for 'speciation genes' in bifurcating speciation (Presgraves 2008; Qvarnström & Bailey 2009; Sætre & Sæther 2010). As noted by Haldane (1922), hybrids of the heterogametic sex typically suffer greater fitness loss than those of the homogametic sex when divergent taxa interbreed. This is mainly because (partially) recessive X- or Z-linked alleles that are incompatible with heterospecific alleles at other loci get exposed to selection in the heterogametic sex but stay masked by dominance in the homogametic sex (Orr 1995, 1997). Sex chromosomes are further characterized by elevated rates of adaptive divergence compared with autosomes, i.e., the faster-X (Z) effect (Charlesworth *et al.* 1987; Mank *et al.* 2007, 2009; Ellegren 2009). Selection will be more effective on the X and Z because partially or fully recessive mutations are not masked by dominance in the heterogametic sex, leading to more effective purging of deleterious mutations and decreased likelihood that favourable mutations get lost by drift (Charlesworth *et al.* 1987). Moreover, the rate of evolution on the Z chromosome will be additionally elevated by any male bias in mutation rate (Axelsson *et al.* 2004; Ellegren 2007). A male-biased mutation rate is expected because mature sperm cells go through more cell divisions than egg cells. This yields a higher mutation rate on the Z because the chromosome spends $^{2}/_{3}$ of its time in the male germ line, compared to the autosomes ½. Finally, sex chromosomes are expected to experience a nonrandom accumulation of genes that affect sex and reproduction (Rice 1984; van Doorn & Kirkpatrick 2007), that is, traits that are important components in both prezygotic and postzygotic barriers to gene flow (Servedio & Sætre 2003). Indeed, both sexual traits and female mate preferences have been found to be sex linked in a number of animal taxa (e.g. Prowell 1998; Reinhold 1998; Ritchie 2000; Noor *et al.* 2001; Iyengar *et al.* 2002; Sætre *et al.* 2003; Sæther *et al.* 2007; Iyengar & Reeve 2009; Pryke 2010).

However, the potential role of sex chromosomes in promoting homoploid hybrid speciation has so far been little discussed (but see Duenez-Guzman *et al.* 2009). We would expect that the parental contributors to a hybrid lineage would be differentiated at Z-linked genes that affect sex and reproduction traits, just as in other species as discussed earlier. Hence, we may hypothesize that recombination between Z-linked parental alleles in a hybrid lineage would be particularly important in creating novel gene combinations that are incompatible with the parental genomes or that

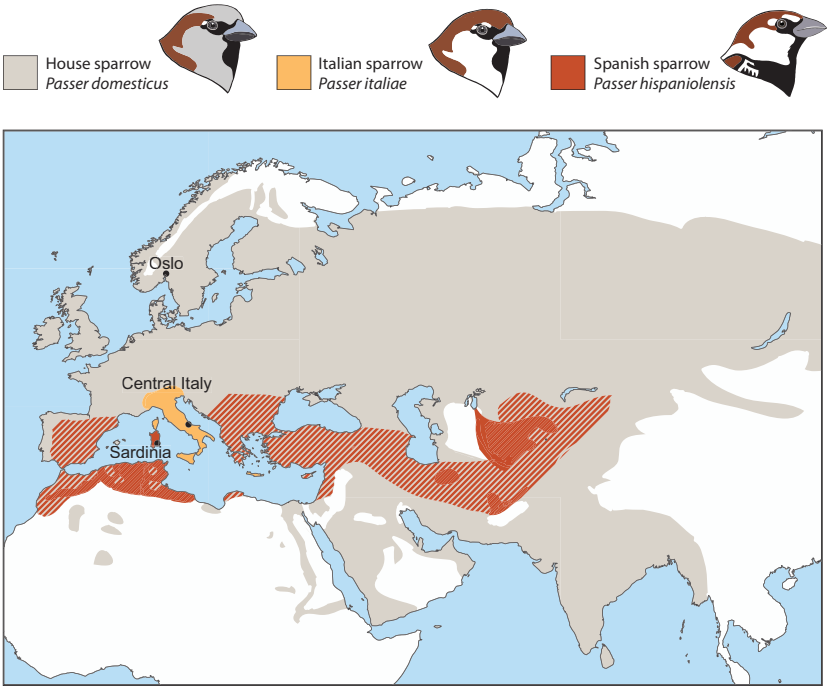

Fig. 1 The Palearctic distribution of the house sparrow (grey), Spanish sparrow (red) and Italian sparrow (yellow). Hatched areas indicate regions where the Spanish sparrow and house sparrow occur sympatrically. Black dots indicate sampling localities.

otherwise function as reproductive barriers against the parental species, including novel secondary sexual characteristics.

Here, we present the results of evolutionary genetic analyses of the Italian sparrow (*Passer italiae*) that has been proposed to be of hybrid origin because males have intermediate plumage characteristics that constitute a mosaic of plumage traits found in the house sparrow (*P. domesticus*) and Spanish sparrow (*P. hispaniolensis*) (see Fig. 1; Zedlitz 1913; Meise 1936; Johnston 1969; Summers-Smith 1988; Töpfer 2006). In an accompanying study (Hermansen *et al.* 2011), we show that the Italian sparrow exhibits clear admixture between its putative parental species at 14 nuclear microsatellites and that it possesses mitochondrial (mt) DNA haplotypes identical to both putative parental species, supporting the hybrid speciation hypothesis. Further, our data indicate that reproductive barriers have developed between the Italian sparrow and both putative parental species (Hermansen *et al.* 2011).

In this study, we analyse variation and divergence at 9 autosomal, 5 Z-linked and one mitochondrial gene in the Italian sparrow and in its putative parental species. According to the hybrid speciation hypothesis, we predict that the Italian sparrow will be genetically intermediate between the house sparrow and Spanish sparrow at both autosomal and Z-linked genes. Further, because of the faster-Z effect, we predict that the putative parental species, the house sparrow and Spanish sparrow, will on average be more differentiated at Z-linked compared with autosomal loci and hence that hybrid genotypes are more likely to be identified as such at the Z-loci in the Italian sparrow. Moreover, faster adaptive divergence on the Z chromosome can lead to a quick accumulation of co-adapted gene complexes that will be incompatible with heterospecific alleles, hence leading to fixations of alternative parental alleles in a hybrid lineage. In contrast, at selectively neutral loci, alleles from both parental species are more likely to be preserved for a longer time in the hybrid lineage. We, therefore, test the prediction that the Z chromosome will show more mosaicism (fixation of alternative parental alleles at different genes) than autosomes in the Italian sparrow.

Finally, owing to the intermediate plumage coloration of the Italian sparrow, we specifically test for possible species-specific differences that correlate with the plumage characteristics of the three taxa by analysing sequence variation at one candidate gene, the *melanocortin-1 receptor* gene (*MC1R*). This gene is involved in the melanin synthesis pathway in vertebrates and has been shown to affect coloration in some birds (Theron *et al.* 2001; Andersson 2003; Doucet *et al.* 2004; Mundy *et al.* 2004).

Materials and methods

Study material

Blood samples of 16 individuals (14 males and 2 females) from each of the three sparrow taxa were collected during spring 2008. We specifically chose individuals from allopatric populations so that population genetic parameters should not be influenced by recent introgression of foreign alleles via hybridization. The Italian sparrows are from Central Italy (Aquaviva-Picena 42°56′43.05″N, 13°48′45.93″E and L′Aquila 42°21′2.66″N, 13°23′59.73″E), the house sparrows are from Norway (Oslo 59°54′49.75″N, 10°44′19.47″E), and the Spanish sparrows are from Sardinia, Italy (Pula 38°58′6.34″N, 08°58′37.14″E) (Fig. 1). In addition to the three focal species, one tree sparrow (*P. montanus*), captured on Sicily, Italy (Montemaggiore Belsito 37°50′50.84″N, 13°45′44.41″E), was included to serve as an outgroup (all coordinates estimated from Google Earth). The birds were caught using mist nets, and 20–50 μL of blood was taken by venipuncture of a brachial vein and stored in a standard buffer. Permissions for catching and sampling birds were obtained for the respective sampling localities by the appropriate authorities in Italy and Norway.

DNA extraction, PCR and sequencing

DNA was extracted using the Molestrips DNA Blood kit together with the GeneMole instrument (Mole Genetics AS, Lysaker, Norway). Sample preparation and DNA isolation were performed according to the manufacturer's recommendations, with minor modifications: 20–40 μL of each blood sample stored in buffer was dissolved with phosphate-buffered saline (PBS) and 4 μL of Proteinase K to a total volume of 200 μL and incubated at 56° with mixing (500 rpm) for at least 2 h before samples were loaded to the instrument.

Numerous primer sets designed and extensively tested in *Ficedula* flycatchers were available from previous studies (Primmer *et al.* 2002; Borge *et al.* 2005; Backström *et al.* 2006, 2008, 2010). The nuclear genetic markers included were chosen based on amplification and sequencing success from a longer list of candidate markers (in total 53 pairs). In addition, we included an exonic sequence of the *melanochortin-1 receptor* gene (*MC1R*) using primers designed by Cheviron *et al.* (2006) (modified from Mundy *et al.* (2004)). Finally, we sequenced the mitochondrial *ND2* gene using primers designed by Sorenson *et al.* (1999) (as modified in http://people.bu.edu/msoren/Bird.mt.Primers.pdf).
The sequences have been deposited in GenBank (accession no's: JF968628–JF969162, JF979448–JF979529, JN029

903–JN029934, JN054301–JN054402, JN090224–JN090512 and JN090588–JN090775). Details on the included markers are summarized in Table 1.

All PCRs were performed in 10 μL volumes containing 4.4 μL mqH_2O, 0.2 μL dNTP (10 mM), 2.0 μL 5 × Phusion® HF Buffer (Finnzymes Oy, Espoo, Finland), 0.5 μL of each primer (10 μM), 0.3 μL 100% DMSO, 0.1 μL Phusion® High-Fidelity DNA Polymerase (Finnzymes) and 2 μL of template DNA. The following PCR profile was used: 98 °C for 30 s (initial denaturation), 98 °C for 8 s (denaturation), 55-67 °C for 30 s (Table 1), 72 °C for 30 s (extension), and then the second through fourth step for another 34–35 cycles before the last step, 72 °C for 30 s (final extension). All PCR products were screened on 2% agarose gels stained with ethidium bromide, and the PCR products were cleaned for excessive primers and nucleotides using ExoSAP-IT (USB Corporation, Cleveland, OH, USA).

Sequencing was performed on an Applied Biosystems 3730XL Analyzer instrument using Big Dye™ Terminator Cycle-Sequencing Kits (Applied Biosystems Inc., Foster City, CA, USA). PCR products were sequenced in both directions using the PCR primers.

Alignment and haplotype identification

All sequences were manually edited and aligned using SEQUENCHER 4.9 (GeneCodes Corp., Ann Arbor, MI, USA). Sequences containing more than one indel were discarded from subsequent analyses. Owing to the presence of multiple indels at some loci or other difficulties in obtaining high-quality sequences, DNA sequences were not analysed for all individuals at all loci.

For sequences with more than one heterozygous site, haplotypes were assigned statistically using PHASE 2.1.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003). This method has been shown to assign haplotypes with high accuracy and may even outperform the more time-consuming and costly alternative of cloning, even in cases of small sample sizes and numerous variable sites

Table 1 Primer information and amplification conditions

Locus	C[1]	Primer sequence (5′–3′)	PCR[2] (°C)	References
ND2	M	F: ACTCTTRTTTAAGGCTTTGAAGGC R: GGCCCATACCCCGRAAATG	58	Sorenson *et al.* (1999)
15743	A	F: CATCCTCAGACCATCATTGC R: AAGTCTTCACGGAACTTCAC	60	Backström *et al.* (2008)
17483	A	F: GAAATGTGGTCTGAACAGTC R: TTGCTCTTGGCACGATATGC	60	Backström *et al.* (2008)
27623	A	F: AAGTGTTACCATGGCAAGAC R: CGCACATTAATTCTCTTGGC	60	Backström *et al.* (2008)
ACLY-16	A	F: ACCATGAATTATCCCCAGGTGAG R: CAAAACCATTGGTACCCCACAG	58	Borge *et al.* (2005)
CKB-2	A	F: GGTTGATAATCCTGGTAAAATGCA R: GATGGCCTGGAGTGGTAATAAAGT	58	Borge *et al.* (2005)
LAMA-2	A	F: CCAAGAAGCAGCTGCAGGATGAGATGC R: CTGCCGCCCGTTGTCGATCTCCACCA	60	Primmer *et al.* (2002)
MC1R	A	F: CTGGCTCCGGAAGGCRTAGAT R: AYGCCAGYGAGGGCAACCA	67	Cheviron *et al.* (2006)
RPL30-3	A	F: CCAAGTTGGTCATCCTAGCCA R: GCCACTATAATGATGGACACCAGTC	63	Borge *et al.* (2005)
TM-5	A	F: CAGCTTCTGTAGCCAGTTAGCTCA R: TGGAAGATTCAGTCAAGCAAAAGA	63	Borge *et al.* (2005)
BRM-15	Z	F: AGCACCTTTGAACAGTGGTT R: TACTTTATGGAGACGACGGA	58	Borge *et al.* (2005)
CHD1Z	Z	F: TAGAGAGATTGAGAACTACAGT R: GACATCCTGGCAGAGTATCT	53	Borge *et al.* (2005)
PLAA	Z	F: CCTGTATCTCCTCGGCACTT R: GTTCAAACAATCAGACTCCC	60	Backström *et al.* (2010)
PPWD1	Z	F: AACTGTGGAAAACTTCTGTG R: TCATCTTCAAATTCTCCTCC	60	Backström *et al.* (2006)
VLDLR	Z	F: CAGAAGTGGAGAATGCATAG R: ACAGTCACATTCATAGCCA	60	Borge *et al.* (2005)

[1]Chromosome class. M, mitochondrial; A, autosomal; Z, Z-linked.
[2]Annealing temperatures.

(Harrigan *et al.* 2008). The analyses were run separately for each species at all loci where more than one heterozygous site were detected, using 10 000 iterations, a thinning interval set to 1, and burnin set to 1000 (see PHASE 2.1 documentation for details). For each locus (and separately for each species), we applied the algorithm five times using different random seeds to assure consistency across independent runs. All other parameters were set to default. The inferred haplotypes were then assigned to the respective individuals. The haplotype reconstruction in PHASE 2.1 yielded consistent assignments across all five runs for most heterozygous sites. In cases of inconsistency (three sites in two loci), we chose the gametic phase inferred by the majority of the runs.

Population genetics and phylogenies

Basic population genetic parameter estimates on polymorphism, divergence and recombination were computed using DNASP 5.0 (Librado & Rozas 2009). Haplotype diversity (H_d) (Nei 1987), nucleotide diversity (π) (Nei & Li 1979), number of polymorphic (segregating) sites (S), average number of nucleotide differences between pairs of sequences (Π), and Tajima's D (Tajima 1989) were computed for each species at all loci. Additionally, for each species pair, we calculated number of shared polymorphisms (S_s), fixed differences (S_f) and average number of nucleotide substitutions per site (D_{xy}) using DNASP 5.0, as well as pairwise F_{ST}-estimates (Weir & Cockerham 1984) using ARLEQUIN 3.5 (Excoffier & Lischer 2010).

Neighbor-joining trees (Saitou & Nei 1987) of all unique haplotypes were reconstructed for each locus using MEGA 4.0 (Tamura *et al.* 2007). The trees were made using 2000 bootstrap replications utilizing the maximum composite likelihood method (Tamura *et al.* 2004).

We conducted F_{ST}-outlier tests employing the LOSITAN user interface (Antao *et al.* 2008) of *F*-DIST (Beaumont & Nichols 1996; Beaumont 2005). Mean neutral F_{ST} in the data set was approximated by initially removing loci potentially under selection (99% CI). A second and final run, using all loci, was then conducted using the last computed mean. Autosomal and Z-linked genotypes were analysed in separate runs.

MC1R and plumage characteristics

To test for the possible associations between *MC1R* alleles and plumage characteristics, we identified any nonsynonymous polymorphism in the full data set (i.e. all three taxa) using DNASP. Plumage characteristics of the birds were scored in discrete classes: (i) colour of the crown/nape (grey or chestnut), (ii) colour of the cheek (white or grey), (iii) colour pattern on the back (black-streaked or brown-streaked) and (iv) the presence/absence of black-streaked flanks. Statistical associations between amino acid and plumage variants were tested using 2×2 contingency tables (chi-square tests).

Results

Polymorphism

The levels of nucleotide variation were slightly higher in the Italian sparrow than in the house sparrow and Spanish sparrow (Table 2). The average nucleotide diversity (π) of the autosomal markers was high in all three species, comparable with that found in other passerine birds or higher (Primmer *et al.* 2002; Borge *et al.* 2005; Carling *et al.* 2010; Storchová *et al.* 2010).

Nucleotide variation was lower at the Z-linked loci relative to that of the autosomal loci; the Z/A ratio of π was 0.55 for the house sparrow, 0.45 for the Italian sparrow and 0.28 for the Spanish sparrow, after correcting for the reduced effective population size of sex-linked markers (three-fourths of autosomal markers assuming equal sex ratios). Again the ratios are comparable with those reported in other passerine birds (ranging from 0.33 in the nightingale (*Luscinea megarhynchos*) (Storchová *et al.* 2010) to 0.56 in the collared flycatcher (*Ficedula albicollis*) (Borge *et al.* 2005). The average number of polymorphic sites (per nucleotide) was significantly lower on the Z-linked loci compared with that of the autosomal loci in the Spanish sparrow (*t*-test: Z-linked loci, mean = 0.0057, $N = 5$, and autosomal loci, mean = 0.0269, $N = 9$; $t = 2.75$, $P = 0.018$) and the Italian sparrow (*t*-test: Z-linked loci, mean = 0.0074, $N = 5$, and autosomal loci, mean = 0.0293, $N = 9$, t = 2.44, $P = 0.031$), and nearly significantly lower also in the house sparrow (*t*-test: Z-linked loci, mean = 0.0109, $N = 5$, and autosomal loci, mean = 0.0247, $N = 9$, $t = 1.84$, $P = 0.091$).

None of the Tajima's D tests differed significantly from zero in any of the species at any locus. Overall, the Italian sparrow exhibited more strongly negative Tajima's D values than the Spanish sparrow and especially the house sparrow. Further, stronger negative values were found on the autosomal markers compared with the Z-linked ones in all three taxa.

Z-linked and autosomal differentiation in the putative parental species

The level of differentiation between the putative parental species, the house sparrow and Spanish sparrow, was higher at the Z-linked loci than at the autosomal

Table 2 Polymorphism summaries in house sparrows (*Passer domesticus*), Italian sparrows (*P. italiae*) and Spanish sparrows (*P. hispaniolensis*) (mitochondrial, autosomal and Z-linked markers)

Locus	C[1]	Species	Length (bp)	Exon (bp)	N[2]	H_d[3]	π[4]	S[5]	Π[6]	TD[7]
ND2	M	*P. domesticus*	969	969	16	0.817	0.002	5	1.542	0.076
		P. italiae			15	0.467	0.001	3	0.514	−1.317
		P. hispaniolensis			16	0.892	0.002	10	2.233	−0.966
15743	A	*P. domesticus*	317	0	24	0.819	0.013	12	4.250	0.766
		P. italiae			30	0.582	0.010	13	3.117	−0.165
		P. hispaniolensis			30	0.777	0.013	16	4.145	−0.117
17483	A	*P. domesticus*	487	35	22	0.896	0.007	14	3.418	−0.335
		P. italiae			22	0.918	0.008	15	3.987	−0.112
		P. hispaniolensis			26	0.908	0.009	19	4.274	−0.505
27623	A	*P. domesticus*	528	0	32	0.919	0.008	15	4.419	0.377
		P. italiae			28	0.923	0.007	18	3.783	−0.636
		P. hispaniolensis			32	0.863	0.007	16	3.421	−0.466
ACLY-16	A	*P. domesticus*	282	0	20	0.416	0.016	13	4.437	0.765
		P. italiae			16	0.733	0.026	16	7.067	1.836
		P. hispaniolensis			12	0.727	0.017	12	4.788	0.861
CKB-2	A	*P. domesticus*	439	0	32	0.692	0.003	7	1.458	−0.470
		P. italiae			32	0.597	0.003	6	1.181	−0.581
		P. hispaniolensis			30	0.607	0.002	6	0.876	−1.203
LAMA-2	A	*P. domesticus*	461	48	28	0.683	0.002	2	0.857	1.397
		P. italiae			30	0.703	0.003	9	1.543	−0.998
		P. hispaniolensis			24	0.641	0.002	3	0.891	0.275
MC1R	A	*P. domesticus*	784	784	32	0.895	0.003	13	2.044	−1.195
		P. italiae			30	0.828	0.002	14	1.906	−1.537
		P. hispaniolensis			32	0.837	0.002	12	1.498	−1.601
RPL30-3	A	*P. domesticus*	341	0	26	0.849	0.014	14	4.655	0.640
		P. italiae			30	0.899	0.015	14	5.023	1.406
		P. hispaniolensis			32	0.881	0.015	13	5.109	1.899
TM-5	A	*P. domesticus*	332	0	30	0.287	0.001	1	0.287	0.216
		P. italiae			30	0.193	0.001	3	0.200	−1.732
		P. hispaniolensis			30	0.191	0.001	2	0.195	−1.256
Average autosomal	A	*P. domesticus*	441	—	27.3	0.717	0.007	10.1	2.869	—
		P. italiae			27.6	0.708	0.008	12.0	3.090	—
		P. hispaniolensis			27.6	0.715	0.007	11.0	2.800	—
BRM-15	Z	*P. domesticus*	370	77	30	0.678	0.004	6	1.524	0.018
		P. italiae			28	0.677	0.004	8	1.519	−0.807
		P. hispaniolensis			30	0.549	0.002	2	0.618	0.461
CHD1Z-15	Z	*P. domesticus*	446	0	30	0.000	0.000	0	0.000	—
		P. italiae			28	0.389	0.001	1	0.389	0.819
		P. hispaniolensis			30	0.297	0.001	2	0.306	−0.808
PLAA	Z	*P. domesticus*	501	0	30	0.343	0.001	4	0.487	−1.328
		P. italiae			27	0.681	0.002	3	0.849	0.220
		P. hispaniolensis			30	0.067	0.000	1	0.067	−1.147
PPWD1	Z	*P. domesticus*	565	0	26	0.760	0.004	7	2.326	0.821
		P. italiae			30	0.825	0.005	8	2.593	0.863
		P. hispaniolensis			30	0.625	0.004	6	2.000	0.915
VLDLR-7	Z	*P. domesticus*	500	0	30	0.844	0.006	9	3.051	1.065
		P. italiae			30	0.657	0.003	7	1.310	−0.764
		P. hispaniolensis			30	0.680	0.002	3	0.844	0.269
Average sex linked	Z	*P. domesticus*	476	—	29.2	0.525	0.003	5.2	1.478	—
		P. italiae			28.6	0.646	0.003	5.4	1.332	—
		P. hispaniolensis			30.0	0.444	0.002	2.8	0.767	—

[1]Chromosome class. M, mtDNA; A, autosomal; Z, Z-linked.
[2]Number of individuals (mtDNA) or haploid sequences (all other loci).
[3]Haplotype diversty (Nei 1987).
[4]Average number of nucleotide differences per site between two randomly chosen sequences (Nei 1987).
[5]Number of polymorphic (segregating) sites.
[6]Average number of nucleotide differences per sequence.
[7]Tajima's D (Tajima 1989).

ones (Table 3). Mean F_{ST} was significantly higher between these two taxa on the Z-linked markers (mean F_{ST} = 0.48) compared with that of the autosomal markers (mean F_{ST} = 0.079; Mann–Whitney *U*-test, P = 0.02). F_{ST}-outlier tests identified two of the Z-linked markers as candidates for having been subject to diversifying selection, namely *CHD1Z* and *PLAA* (Fig. 2). All the other loci were inferred to have evolved neutrally.

The house sparrow and Spanish sparrow exhibited fewer shared polymorphisms but more fixed differences at the Z-linked compared with the autosomal markers. In total, the two taxa exhibited 3 fixed substitutions and 9 shared polymorphisms on the Z-linked markers, compared with 0 fixed substitutions and 62 shared polymorphisms on the autosomal ones (Fisher's exact test, P = 0.003). Again, similar patterns have been found in other pairs of passerine sister species (Borge *et al.* 2005; Storchová *et al.* 2010).

Genetic patterns in the Italian sparrow

The Italian sparrow is apparently genetically intermediate to the house sparrow and Spanish sparrow according to both F_{ST}-estimates and D_{xy} (average number of nucleotide substitutions per site between pairs of sequences between taxa) (Table 3). Overall, the F_{ST}-estimates tended to be somewhat higher between the Italian and the house sparrow than between the Italian and the Spanish sparrow (Table 3), but with notable exceptions.

Fixed substitutions between the house sparrow and Spanish sparrow occurred at three loci (Table 3). The Italian sparrow alternately associated with either of the two species at these three loci. At the mitochondrial *ND2* gene, the Italian and house sparrow formed a monophyletic clade (Fig. 3), separated from the Spanish sparrow by 36 fixed substitutions (Table 2). However, F_{ST} was rather high and significantly different from zero also between the Italian sparrow and house sparrow at this gene (Table 3). Closer inspection of the sequences suggests that the relatively high F_{ST}-value stems from different allele frequencies at a single nucleotide site in the two taxa (house sparrows being monomorphic and the alternative allele being the more common one in the Italian sparrows). At the Z-linked *CHD1Z* gene, the opposite pattern of that on *ND2* was found. Here, the Italian sparrow formed a monophyletic clade with the Spanish sparrow (Fig. 3), both being separated from the house sparrow by two fixed substitutions (Table 3). Further, most Italian sparrows possessed the allele that was also the most common one among the Spanish sparrows (Fig. 3). Finally, at the *PLAA* gene (also Z-linked), the Italian sparrows possessed alleles identical to both putative parental species (19 house sparrow derived alleles and 8 Spanish sparrow derived alleles; Table 3, Fig. 3).

MC1R—the candidate gene for colour polymorphism

The candidate gene for colour polymorphism *MC1R* exhibited substantial allelic variation (Table 2), but with little differentiation between the three taxa (Table 3; Fig. 3). Surprisingly, perhaps, the majority of the polymorphic sites were nonsynonymous (NS) and relatively few were synonymous (S). The ratio of S to NS was 2:11 in the house sparrow, 1:11 in the Spanish sparrow and 2:13 in the Italian sparrow. However, the three taxa shared one common (resulting) polypeptide. All the alternative amino acid sequences were closely derived from the most common one and occurred at low frequency in three focal taxa without showing any clear pattern of species association (see also Table 3). Consequently, no association between amino acid variation at *MCIR* and any plumage characteristic (crown/nape colour, cheek colour, back colour or pattern of streaking on flank) was detected ($P > 0.1$ in all comparisons; chi-squared tests).

Discussion

Our results show that the Italian sparrow is genetically intermediate between the house sparrow and the Spanish sparrow, supporting the hybrid origin hypothesis (see our accompanying study, Hermansen *et al.* 2011, for a full discussion of the evidence for hybrid origin vs. alternative explanations in the case of these *Passer* sparrows). Later, we first discuss the pattern of genetic differentiation in the putative parental species, the house sparrow and the Spanish sparrow. Thereafter, we discuss the genetics of the Italian sparrow in relation to the hybrid origin hypothesis in greater detail.

Pattern of polymorphism and divergence in the putative parental species

The standard neutral expectation is that the level of intraspecific variation and interspecific divergence should be positively correlated across loci (e.g. Li & Graur 2006). In contrast, we found that the level of polymorphism was substantially lower at Z-linked compared with that autosomal loci in the house sparrow and Spanish sparrow, well below what could be expected from the lower effective population size of Z-linked genes compared to autosomal ones (0.75). Yet, the Z-linked markers showed higher degree of divergence between the two taxa and fewer shared polymorphism than the autosomal ones.

Table 3 Divergence estimates and number of fixed and shared polymorphisms between three taxa of *Passer* sparrows (mitochondrial, autosomal and Z-linked markers)

Locus	C[1]	Species pairs		S_s[2]	S_f[3]	D_{xy}[4]	F_{ST}	*P*-values
ND2	M	*P. domesticus*	*P. italiae*	0	0	0.0018	0.392	***
		P. hispaniolensis	*P. italiae*	0	36	0.0405	0.964	***
		P. domesticus	*P. hispaniolensis*	1	36	0.0403	0.952	***
15743	A	*P. domesticus*	*P. italiae*	11	0	0.0123	0.057	
		P. hispaniolensis	*P. italiae*	12	0	0.0111	−0.023	
		P. domesticus	*P. hispaniolensis*	10	0	0.0138	0.040	
17483	A	*P. domesticus*	*P. italiae*	12	0	0.0087	0.122	**
		P. hispaniolensis	*P. italiae*	12	0	0.0087	0.030	*
		P. domesticus	*P. hispaniolensis*	13	0	0.0093	0.145	***
27623	A	*P. domesticus*	*P. italiae*	11	0	0.0077	0.049	*
		P. hispaniolensis	*P. italiae*	13	0	0.0069	0.009	
		P. domesticus	*P. hispaniolensis*	6	0	0.0076	0.077	**
ACLY-16	A	*P. domesticus*	*P. italiae*	12	0	0.0223	0.101	
		P. hispaniolensis	*P. italiae*	12	0	0.0248	0.117	
		P. domesticus	*P. hispaniolensis*	8	0	0.0172	0.127	*
CKB-2	A	*P. domesticus*	*P. italiae*	5	0	0.0030	−0.013	
		P. hispaniolensis	*P. italiae*	4	0	0.0023	−0.014	
		P. domesticus	*P. hispaniolensis*	4	0	0.0027	0.010	
LAMA-2	A	*P. domesticus*	*P. italiae*	2	0	0.0028	0.059	*
		P. hispaniolensis	*P. italiae*	2	0	0.0027	0.015	
		P. domesticus	*P. hispaniolensis*	2	0	0.0019	−0.019	
MC1R	A	*P. domesticus*	*P. italiae*	9	0	0.0025	−0.002	
		P. hispaniolensis	*P. italiae*	8	0	0.0022	0.002	
		P. domesticus	*P. hispaniolensis*	8	0	0.0024	0.043	*
RPL30-3	A	*P. domesticus*	*P. italiae*	12	0	0.0165	0.138	***
		P. hispaniolensis	*P. italiae*	12	0	0.0151	0.015	
		P. domesticus	*P. hispaniolensis*	11	0	0.0178	0.192	***
TM-5	A	*P. domesticus*	*P. italiae*	1	0	0.0008	0.046	
		P. hispaniolensis	*P. italiae*	1	0	0.0006	−0.011	
		P. domesticus	*P. hispaniolensis*	0	0	0.0008	0.095	*
All autosomal loci[5]	A	*P. domesticus*	*P. italiae*	75	0	0.0085	0.062	
		P. hispaniolensis	*P. italiae*	76	0	0.0083	0.015	
		P. domesticus	*P. hispaniolensis*	62	0	0.0082	0.079	
BRM-15	Z	*P. domesticus*	*P. italiae*	6	0	0.0047	0.131	***
		P. hispaniolensis	*P. italiae*	2	0	0.0031	0.071	*
		P. domesticus	*P. hispaniolensis*	1	0	0.0035	0.183	***
CHD1Z-15	Z	*P. domesticus*	*P. italiae*	0	2	0.0050	0.916	***
		P. hispaniolensis	*P. italiae*	1	0	0.0008	0.008	
		P. domesticus	*P. hispaniolensis*	0	2	0.0049	0.929	***
PLAA	Z	*P. domesticus*	*P. italiae*	1	0	0.0015	0.080	*
		P. hispaniolensis	*P. italiae*	0	0	0.0018	0.457	***
		P. domesticus	*P. hispaniolensis*	0	1	0.0026	0.713	***
PPWD1	Z	*P. domesticus*	*P. italiae*	6	0	0.0054	0.185	***
		P. hispaniolensis	*P. italiae*	5	0	0.0041	0.002	
		P. domesticus	*P. hispaniolensis*	5	0	0.0055	0.299	***
VLDLR7	Z	*P. domesticus*	*P. italiae*	6	0	0.0051	0.172	**
		P. hispaniolensis	*P. italiae*	2	0	0.0023	0.063	**
		P. domesticus	*P. hispaniolensis*	3	0	0.0053	0.278	***
All sex-linked loci[5]	Z	*P. domesticus*	*P. italiae*	19	2	0.0044	0.297	
		P. hispaniolensis	*P. italiae*	10	0	0.0024	0.120	
		P. domesticus	*P. hispaniolensis*	9	3	0.0043	0.480	

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

[1]Chromosome class. M, mitochondrial; A, autosomal; Z, Z-linked.

[2]Number of shared mutations (polymorphisms) between taxa.

[3]Number of fixed differences between taxa.

[4]Average number of nucleotide substitutions per site between taxa.

[5]The sum is shown for S_s and S_f. The mean is shown for D_{xy} and F_{ST}.

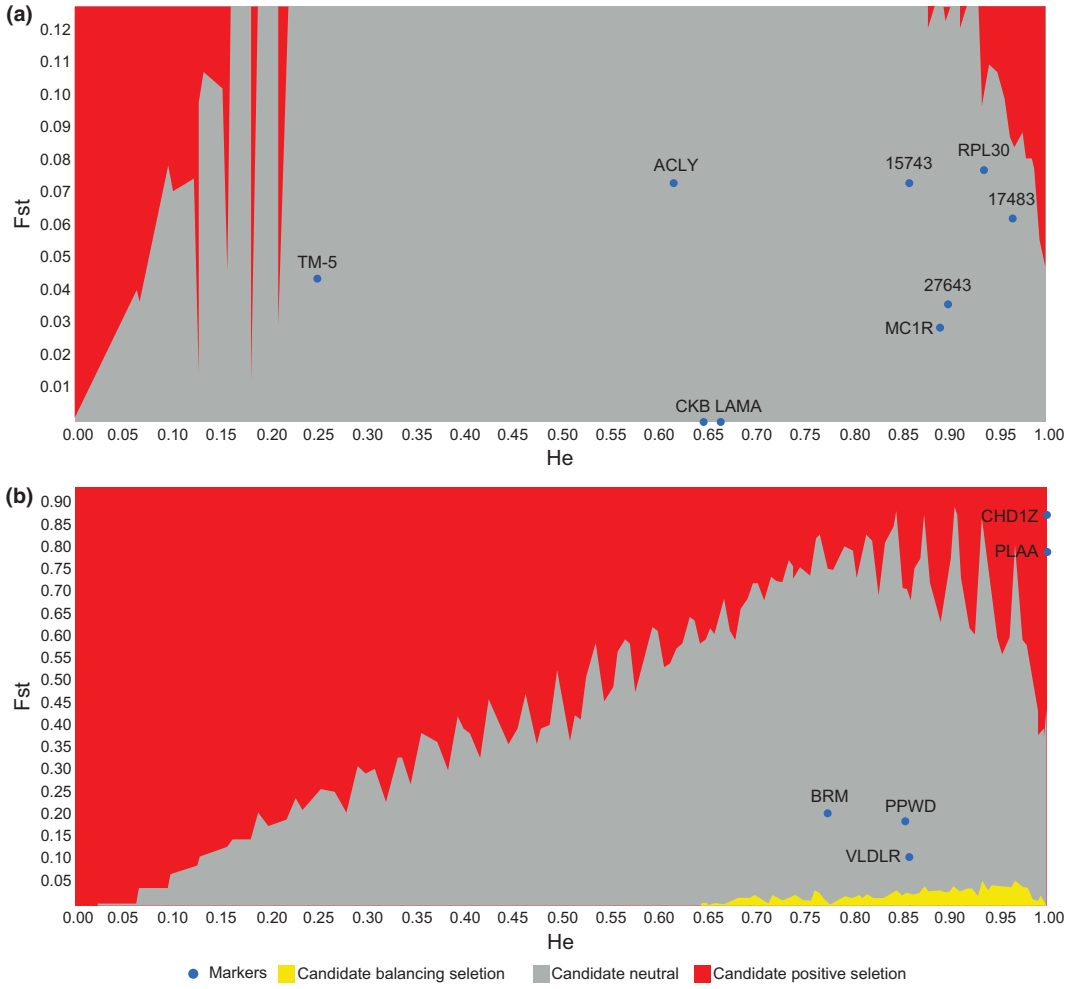

Fig. 2 Results from F_{ST}-outlier tests of (a) autosomal and (b) Z-linked loci between house sparrows and Spanish sparrows. Blue dots indicate F_{ST}-estimates against expected heterozygosity (H_e) of the individual loci. The coloured areas represent the estimated parameter space of candidate positive selection, neutral evolution and balancing selection as indicated.

A similar pattern of apparently nonneutral reduced variation but elevated divergence on the Z chromosome has been reported in other bird taxa (Sundström *et al.* 2004; Borge *et al.* 2005; Storchová *et al.* 2010). Two main explanations have been suggested to account for this pattern. First, the faster-Z (or X) effect encompasses a faster rate of adaptive evolution on the Z chromosome, owing to the hemizygous exposure of beneficial recessive (or partially recessive) alleles in the heterogametic sex (Charlesworth *et al.* 1987). Associated selective sweeps would tend to increase the rate of interspecific divergence and reduce intraspecific variation at linked sites (Sætre *et al.* 2003; Servedio & Sætre 2003; Sundström *et al.* 2004; Borge *et al.* 2005; Mank *et al.* 2007, 2009). Hemizygosity would likewise expose deleterious (partially) recessive mutations to purging, which would reduce intraspecific variation even further (Charlesworth *et al.* 1987; Mank *et al.* 2009). Second, the pattern can emerge as an indirect consequence of Haldane's rule (Sætre *et al.* 2003; Coyne & Orr 2004; Borge *et al.* 2005; Carling *et al.* 2010; Storchová *et al.* 2010). Reduced fitness of heterogametic hybrids caused by exposure of (partially) recessive incompatibility loci would be associated with reduced introgression on the Z chromosome. However, the two hypotheses are not mutually exclusive. For instance, a faster-Z effect may increase the number of loci that are incompatible between the species and thus contribute to a reduced introgression rate of Z-linked genes (Sætre *et al.* 2003; Coyne & Orr 2004).

In the case of the house sparrow and Spanish sparrow, we found evidence compatible with the faster-Z hypothesis. Two of the Z-linked genes in this study (*CHD1Z* and *PLAA*) turned out as candidates of positive, divergent selection according to the F_{ST}-outlier test. On the other hand, genes subject to divergent selection may also introgress at a lower rate than

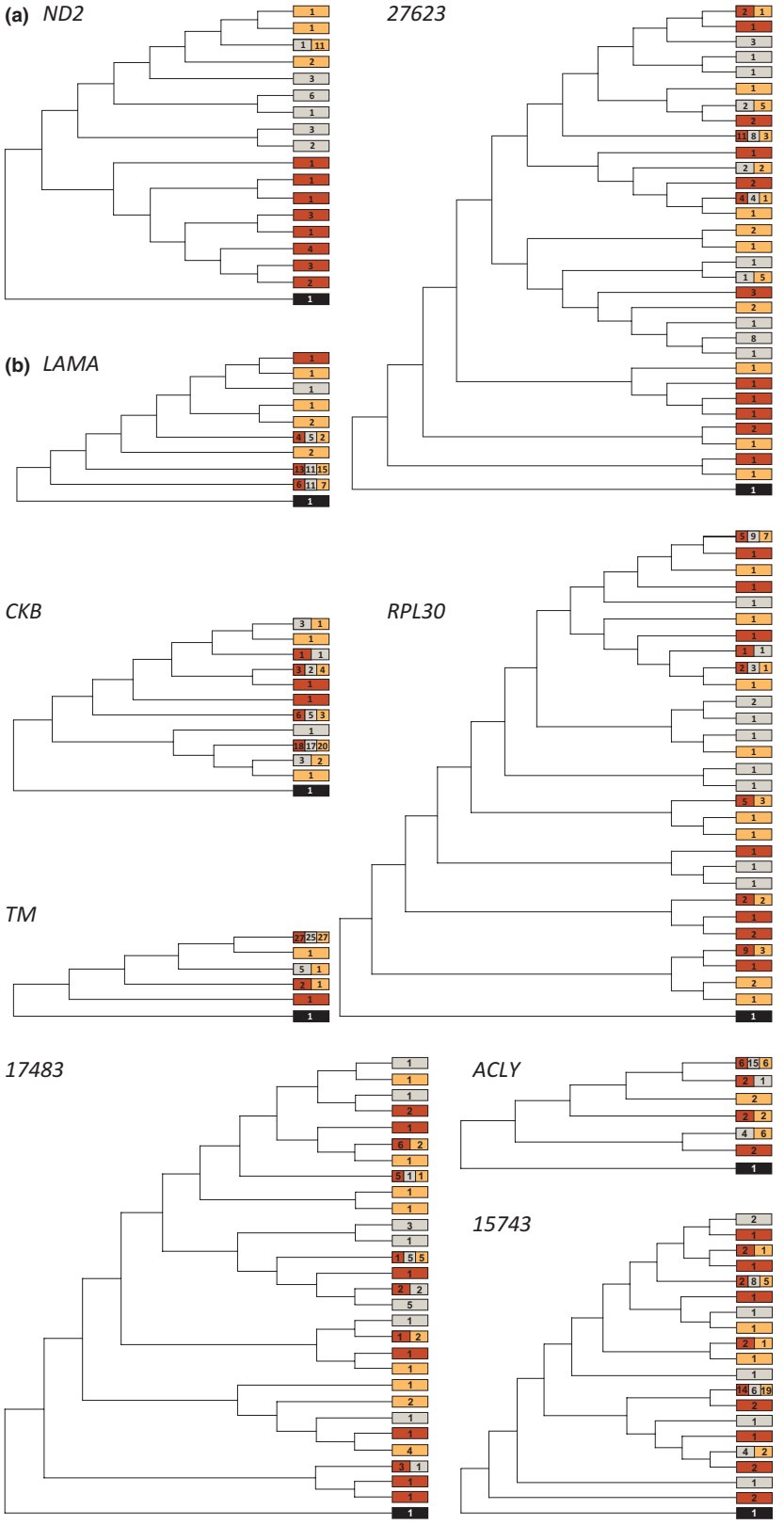

Fig. 3 Neighbour-joining trees (topology display) for (a) one mtDNA, (b) nine autosomal and (c) five Z-linked loci for house sparrows (*Passer domesticus*), Italian sparrows (*P. italiae*) and Spanish sparrow (*P. hispaniolensis*). The tree sparrow (*P. montanus*) was used as outgroup. The terminal branches represent unique haplotypes. The coloured boxes indicate species and the number of individuals.

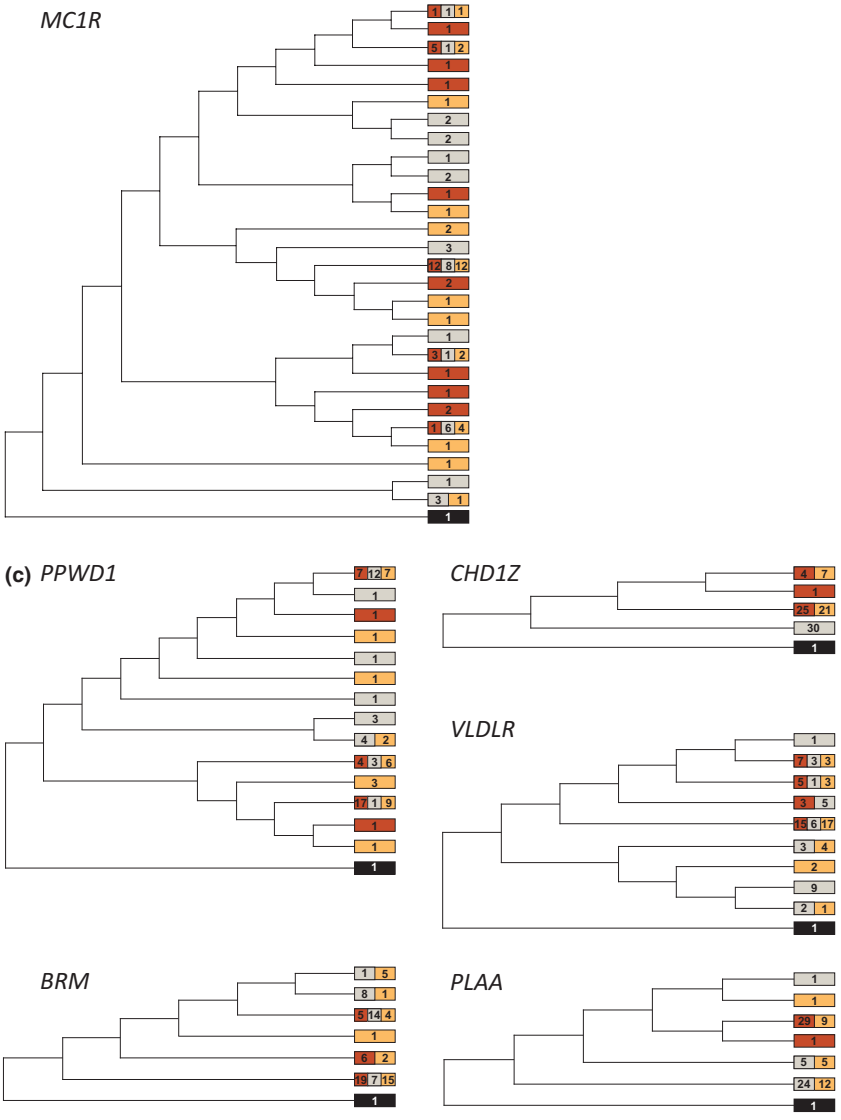

Fig. 3 (*Continued*).

neutral loci. Hence, our results do not rule out differential introgression as a contributing explanation for the different pattern of polymorphism and divergence on Z-linked vs. autosomal loci. Interestingly, both *CHD1Z* and *PLAA* turned out as outliers also in a study on *Ficedula* flycatchers (Backström *et al.* 2010). As for the former gene, *CHD* proteins are thought to have a role in gene expression by remodelling the chromatin structure and may thus alter the expression of several genes (Stokes & Perry 1995; Lee *et al.* 2002; Agate *et al.* 2004). Accordingly, it is tempting to speculate that *CHD1Z* may commonly play a role in species differentiation and consequently in promoting reproductive isolation.

To sum up so far, our results suggest a role for hemizygosity in reducing intraspecific variation yet elevating interspecific divergence on the Z chromosome relative to autosomes in the house sparrow and Spanish sparrow. However, further studies utilizing more markers (more statistical power) are needed to disentangle the relative importance of a faster-Z effect *per se* and differential introgression. More markers and statistical power would enable us to estimate key parameters, such as the amount of gene flow of Z-linked vs. autosomal markers, which can be used to directly address key predictions of the two hypotheses.

The Z chromosome and homoploid hybrid speciation

Given the predominant role of the Z chromosome in bifurcating speciation (see e.g. Qvarnström & Bailey 2009 for a recent review), we suggest that sex chromosome

evolution might also be instrumental for our understanding of hybrid speciation. Specifically, we predicted that the Italian sparrow (i) would be genetically intermediate between the putative parental species at both Z-linked and autosomal loci, (ii) would show clearer signs of being of mixed ancestry on the Z chromosome owing to greater divergence between the parental taxa and (iii) would show more mosaicism on the Z chromosome because of the faster-Z effect and accumulation of co-adapted gene complexes that would be incompatible with heterospecific alleles. Indeed, according to the divergence and distance estimates, the Italian sparrow appears to be genetically intermediate between both suggested parental species at both the Z-linked and autosomal loci investigated. Moreover, the degree of divergence was more prevalent on the Z-linked genes compared with that on the autosomal genes in the putative parental species. Consequently, whereas quite a few autosomal markers were essentially uninformative for evaluating the potential hybrid status of the Italian sparrow (as all three taxa were undifferentiated), the Z-linked markers showed marked differentiation between the house sparrow and Spanish sparrow, which facilitated the identification of hybrid genotypes in the Italian sparrow. Finally, we found evidence for mosaicism on the Z chromosome as the Italian sparrow only possessed Spanish sparrow alleles on *CHD1Z* but mostly house sparrow alleles on *PLAA*. Although we have not included a sufficient number of markers in this study to test whether this represents a general elevated degree of mosaicism, we find it alluring that both the latter markers stood out as candidates for positive selection according to the F_{ST}-outlier test.

At the mitochondrial *ND2* gene, the Italian and house sparrow formed a monophyletic clade, both separated by 36 substitutions from the Spanish sparrow. This is opposite to the result on the Z-linked *CHD1Z* locus where the Italian sparrows clustered with the Spanish sparrow, hence adding to the picture of a mosaic genome. In our accompanying study, a larger number of individuals were genotyped for (a shorter alignment of) *ND2* (Hermansen *et al.* 2011). In the larger sample, we found one individual possessing a Spanish sparrow haplotype (40 had house sparrow haplotypes), suggesting that the Italian sparrow is not entirely fixed for house sparrow–derived mitochondria.

Comparison of birds with Lepidopterans is of special interest because they share the same sex determining system, i.e., female heterogamety (males ZZ, females ZW; Bull 1983). Further, several Lepidopteran species have been proposed to be of hybrid origin (Scriber & Ording 2005; Gompert *et al.* 2006; Mavárez *et al.* 2006; Mavárez & Linares 2008). Moreover, several traits related to reproductive isolation have been mapped to the Z chromosome also in Lepidoptera (Prowell 1998; Iyengar & Reeve 2009). So far, large-scale comparisons of autosomal and sex-linked markers have to our knowledge not been conducted in any of the proposed examples of hybrid speciation among Lepidopteran species. Yet, in a methodologically similar study to the present one, Salazar *et al.* (2008) investigated one sex-linked and four autosomal markers to determine the relationship between *H. heurippa* and its proposed parental species, *H. melpomene* and *H. cydno*. Interestingly, the Z-linked *Tpi* gene was the only marker that clearly separated all the three taxa, while at the four autosomal loci, the three taxa exhibited considerable allele sharing. Also, *Tpi* has been demonstrated to associate with sterility in hybrid backcrosses involving *H. melpomene* (Jiggins *et al.* 2001). Hence, circumstantial evidence point to a potentially very interesting role for sex chromosomes also in hybrid speciation.

Plumage coloration

Sequence analysis of the coding *MC1R* gene (autosomal) did not reveal any clear association, neither with plumage traits nor between the three taxa. Sequence variation at this gene has been shown to associate with melanic plumage polymorphisms in some bird taxa (Theron *et al.* 2001; Andersson 2003; Doucet *et al.* 2004; Mundy *et al.* 2004), whereas no association has been found in others (MacDougall-Shackleton *et al.* 2003; Cheviron *et al.* 2006). Thus, other genes than *MC1R* probably underlie the plumage differences in the latter species. Indeed, numerous genes have been shown to be involved in pigmentation in vertebrates (Bennett & Lamoreux 2003; Hoekstra 2006; Hoekstra & Coyne 2007). Interestingly, two such 'colour genes' (*SLC45A2* and *TYRP1*), both Z-linked, were identified as candidates for positive, divergent selection in a study on *Ficedula* flycatchers (Backström *et al.* 2010). Plumage colour is often the target of mate choice and sexual selection in birds and may thus be important in promoting premating isolation (see e.g. Sætre *et al.* 1997; Pryke 2010). Accordingly, one may hypothesize that it has been instrumental in the origin of the hybrid Italian sparrow as well. Therefore, sequence analysis of other candidate genes known to be involved in the melanin synthesis, including the two Z-linked ones investigated by Backström *et al.* (2010), will be followed up by future research.

Conclusion

Here, we provide the first investigation of a possible role for sex chromosomes in homoploid hybrid speciation. The Italian sparrow is shown to possess a mixed

nuclear genome resulting from substantial genetic contribution from both suggested parental species, the house sparrow and Spanish sparrow. Further, the suggested parental species were found to be more differentiated at the Z chromosome relative to the autosomes, a finding that is compatible both with the faster-Z (X) effect and/or reduced introgression on the Z chromosome. In accordance with this, we also found clearer signals of the Italian sparrow being of mixed ancestry, and apparently more mosaicism, at the Z chromosome relative to the autosomes in the putative hybrid lineage. Hence, the recombination between diverged Z-linked parental alleles in the hybrid lineage may be of particular importance in creating novel gene combinations that yield reproductive barriers against the parental species. We thus suggest a targeted focus on sex chromosome evolution in future studies of suspected cases of homoploid hybrid speciation.

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The authors share a common interest in evolutionary biology with a special focus on the role of hybridization in evolution. Topics of interest include hybrid speciation, reinforcement, sexual selection and phylogeography.

Data accessibility

DNA sequences: Genebank accessions JF968628–JF969162, JF979448–JF979529, JN029903–JN029934, JN054301–JN054402, JN090224–JN090512 and JN090588–JN090775.