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Hybrid ancestry of an island subspecies of Galápagos mockingbird explains discordant gene trees

Pirmin Nietlisbach [a,*], Peter Wandeler [a], Patricia G. Parker [b], Peter R. Grant [c], B. Rosemary Grant [c], Lukas F. Keller [a], Paquita E.A. Hoeck [a,1]

[a] Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
[b] Department of Biology, University of Missouri–St. Louis, R223 Research Building, One University Boulevard, St. Louis, MO 63121, USA
[c] Department of Ecology and Evolutionary Biology, Princeton University, 106A Guyot Hall, Princeton, NJ 08544, USA

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ABSTRACT

Introgression of genes through hybridization has been proposed to be an important driver of speciation, but in animals this has been shown only in relatively few cases until recently. Additionally, introgressive hybridization among non-sister species leads to a change in the gene tree topology of the concerned loci and thus complicates phylogenetic reconstruction. However, such cases of ancient introgression have been very difficult to demonstrate in birds. Here, we present such an example in an island bird subspecies, the Genovesa mockingbird (*Mimus parvulus bauri*). We assessed phylogenetic relationships and population structure among mockingbirds of the Galápagos archipelago using mitochondrial and nuclear DNA sequences, autosomal microsatellites, and morphological measurements. Mitochondrial haplotypes of Genovesa mockingbirds clustered closely with the haplotypes from two different species, San Cristóbal (*M. melanotis*) and Española (*M. macdonaldi*) mockingbirds. The same pattern was found for some haplotypes of two nuclear gene introns, while the majority of nuclear haplotypes of Genovesa mockingbirds were shared with other populations of the same species (*M. parvulus*). At 26 autosomal microsatellites, Genovesa mockingbirds grouped with other *M. parvulus* populations. This pattern shows that Genovesa mockingbirds contain mitochondria and some autosomal alleles that have most likely introgressed from *M. melanotis* into a largely *M. parvulus* background, making Genovesa mockingbirds a lineage of mixed ancestry, possibly undergoing speciation. Consistent with this hypothesis, mockingbirds on Genovesa are more clearly differentiated morphologically from other *M. parvulus* populations than *M. melanotis* is from *M. parvulus*.

1. Introduction

Speciation is the key process creating the astonishing biological diversity that can be found on earth. Speciation may happen through several routes, including hybridization (Schwenk et al., 2008; Butlin et al., 2009). Although the potential importance of hybridization for speciation in plants has long been recognised, relatively few examples were known to exist in animals until recently (Arnold, 2006; Mallet, 2007; Mavárez and Linares, 2008; Schwenk et al., 2008). For example, hybridization appears to be one of the reasons for the rapid speciation in African cichlid fish (Salzburger et al., 2002; Seehausen, 2004, 2006) and for the adaptive radiation of Darwin's finches (Grant and Grant, 2008a,b, 2009). Apart from its role in speciation, hybridization can cause complications when attempting to reconstruct phylogenies, especially when analysing only few genetic loci (Funk and Omland, 2003). If hybridization among non-sister lineages is at some loci followed by the replacement of alleles in one lineage by alleles from the other lineage, the topology of the gene trees of these loci does not reflect the species tree (Rheindt and Edwards, 2011). Although this phenomenon of ancient introgression is potentially widespread, it has been extremely difficult to conclusively demonstrate in birds (Peters et al., 2007; Rheindt and Edwards, 2011; Warren et al., 2012). Here we present a case of such topology-changing ancient introgressive hybridization in Galápagos mockingbirds and discuss its potential contribution to morphological lineage divergence and speciation.

The Galápagos Islands and their mockingbirds have become famous due to their key role in Darwin's formulation of his theory of evolution by natural selection (Chancellor and Keynes, 2006): Darwin's Galápagos mockingbird specimens triggered his ideas about adaptive radiation, with a mainland form speciating into

* Corresponding author.
E-mail addresses: pirmin.nietlisbach@ieu.uzh.ch (P. Nietlisbach), peter.wandeler@ieu.uzh.ch (P. Wandeler), pparker@umsl.edu (P.G. Parker), prgrant@princeton.edu (P.R. Grant), rgrant@princeton.edu (B.R. Grant), lukas.keller@ieu.uzh.ch (L.F. Keller), phoeck@sandiegozoo.org (P.E.A. Hoeck).
[1] Current address: Institute for Conservation Research, San Diego Zoo Global, 15600 San Pasqual Valley Road, Escondido, CA 92027, USA.

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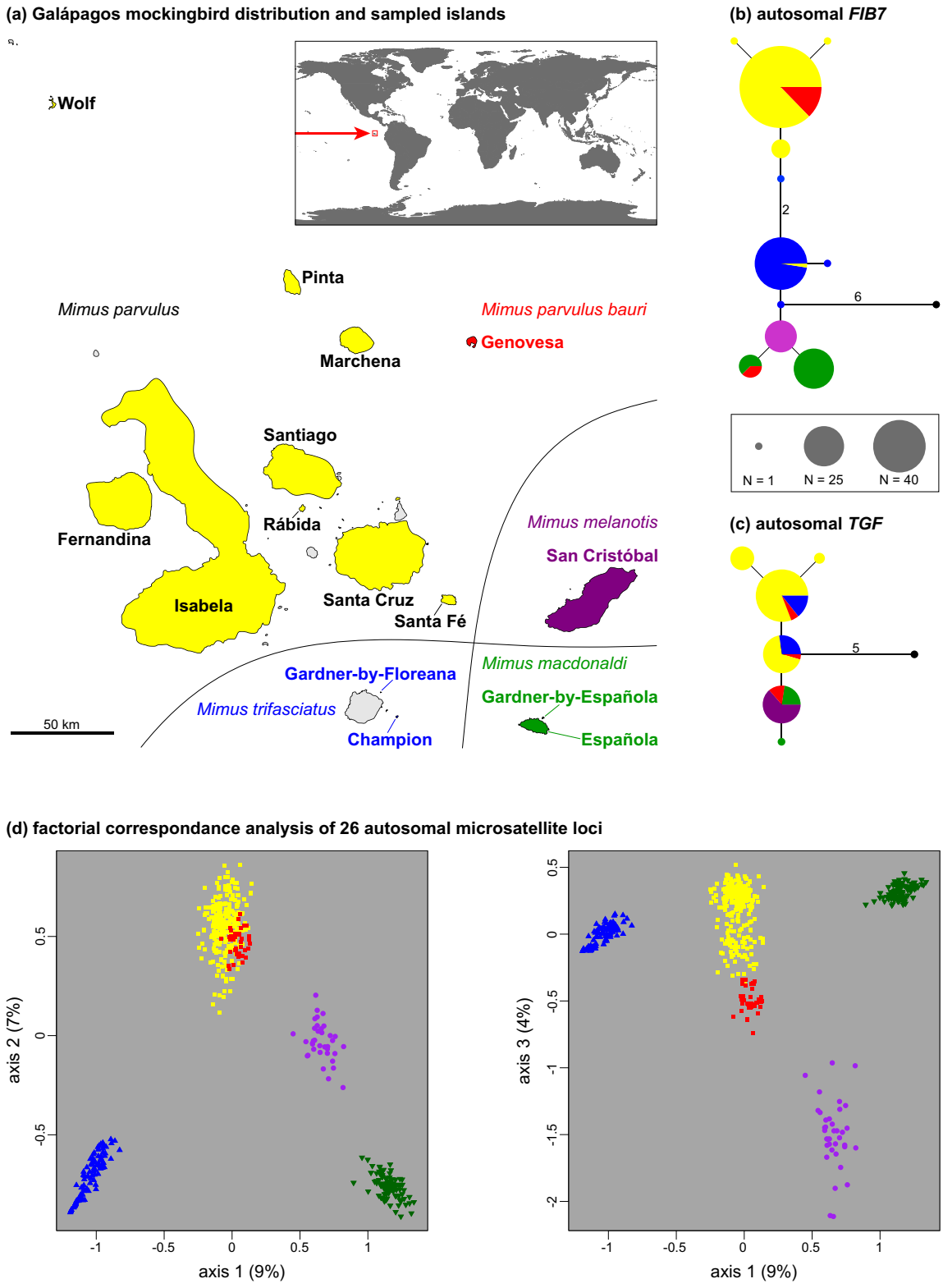

Fig. 1. Autosomal genetic data. (a) Map of the Galápagos archipelago with the distribution of the four recognised mockingbird species and colour-coded islands sampled in this study (for sample sizes see Table 2). (b) and (c) Median-joining networks for the nuclear introns *FIB7* and *TGF*, respectively. Pies represent haplotypes and are coloured according to taxon (see Fig. 1a; black pies represent the outgroup *Mimus longicaudatus*) and sized relative to the abundance of the respective haplotype (see grey reference pies; note that *N* represents the number of haplotypes, not the number of individuals). Numbers along lines represent the number of mutations occurring between two nodes, if higher than 1. Note that for both datasets, individuals from Genovesa display haplotypes grouping with San Cristóbal and Española, as well as with other *M. parvulus*. (d) Plots of the first three axes of a factorial correspondence analysis of 26 microsatellite loci. Note that Genovesa groups among the other *M. parvulus* populations.

several island forms upon colonisation of the archipelago (Darwin, 1839). The Galápagos archipelago, situated in the Pacific Ocean 960 km west of mainland Ecuador, is inhabited by several mockingbird taxa. Their taxonomy has experienced various changes over the last century, but based on phenotypes mockingbirds in Galápagos are currently considered to form four different species (Fig. 1a;

Swarth, 1931; Remsen et al., 2012). After extinction on the large island of Floreana around the year 1880, the Floreana mockingbird, *Mimus trifasciatus*, is now restricted to the two small islets Champion and Gardner-by-Floreana (Curry, 1986; Grant et al., 2000). The Española mockingbird, *M. macdonaldi*, inhabits Española and its adjacent islet Gardner-by-Española. The San Cristóbal mockingbird, *M. melanotis*, is restricted to San Cristóbal Island, whereas most islands in the north-west are inhabited by a widespread fourth species, *M. parvulus*, which is split into several subspecies. Plumage differs among these species (Swarth, 1931; Cody, 2005). Floreana mockingbirds show large dusky patches on each side of the breast and dark spots across it, and a whitish auricular region. Española mockingbirds have a band of brown blotches across the breast and a dusky auricular region, while San Cristóbal mockingbirds also have a dusky auricular patch and no or indistinct spotting on the breast. The fourth species, *M. parvulus*, has a whitish unmarked breast and a black auricular region. Most of its subspecies show a rather dark back and crown, while the subspecies on Genovesa and Santa Fé have a lighter back with more distinct streaks or spots and a greyish crown (Swarth, 1931; Cody, 2005). These allopatric species also differ in morphological measurements (Swarth, 1931; Bowman and Carter, 1971; Abbott and Abbott, 1978; Curry, 1989; Curry and Grant, 1990), as well as in social organisation (Curry, 1989), song (Gulledge, 1970), and foraging behaviour (Bowman and Carter, 1971; Curry and Anderson, 1987). Furthermore, they rarely disperse among islands (P.R. Grant and B.R. Grant, own observations; R.L. Curry, personal communication) and thus populations on different islands are genetically well differentiated (Hoeck et al., 2010).

Arbogast et al. (2006) investigated the phylogeny of Galápagos mockingbirds and their non-Galápagos relatives using molecular techniques. Their mitochondrial DNA (mtDNA) sequence data suggested a single colonisation event of the islands followed by diversification within the archipelago, and revealed an unexpected pattern: four lineages of mockingbirds in the Galápagos were identified that could be considered distinct at the species level, but these four lineages did not correspond to the four phenotypically defined species (described above). Instead, the 7 examined mockingbird individuals from Genovesa Island (*M. parvulus bauri*) clustered closely with the 13 birds from Española (*M. macdonaldi*) and the 2 from San Cristóbal (*M. melanotis*), i.e. birds phenotypically classified as belonging to three distinct species. Four different scenarios explaining the discrepancy between morphology-based taxonomy and mitochondrial genealogy were put forward by the authors. (i) The mitochondrial gene tree reflects the species tree, with extensive morphological convergence explaining the similarity of Genovesa mockingbirds with *M. parvulus* from other islands, leading to their designation as a subspecies of *M. parvulus*. (ii) The plumage of *M. parvulus* represents the ancestral state, while *M. macdonaldi* and *M. melanotis* derived their characteristic plumage only recently. Under this scenario, Genovesa mockingbirds are identified as *M. parvulus* due to plumage symplesiomorphies, while they actually are more closely related to *M. macdonaldi* and *M. melanotis* than to other populations of *M. parvulus*. Under both scenarios (i) and (ii), the mitochondrial gene tree reflects the species tree and hence, mitochondrial as well as nuclear genes should reveal the same clades. Alternatively, two scenarios of differential introgression at nuclear and mitochondrial loci could produce patterns where the mitochondrial gene tree does not reflect the species phylogeny. (iii) Genovesa mockingbirds are most closely related to *M. melanotis* and *M. macdonaldi*, but nuclear loci affecting morphology have entered the Genovesa population via male-mediated dispersal from other *M. parvulus* populations without a corresponding influx of maternal mtDNA. Under this scenario, mtDNA and most nuclear genes should show a grouping of Genovesa mockingbirds with *M. melanotis* and *M. macdonaldi*, while some nuclear genes would group Genovesa mockingbirds with other *M. parvulus* populations. (iv) Genovesa mockingbirds are most closely related to *M. parvulus* but dispersal of *M. melanotis* females from San Cristóbal has replaced the ancestral mtDNA lineages in the Genovesa population. As this scenario requires female immigrants, it is compatible with the generally female-biased dispersal in birds (Greenwood and Harvey, 1982), including, on a small geographic scale, Genovesa mockingbirds (Curry and Grant, 1989). Under such a scenario, the majority, but not necessarily all, of the nuclear genes would show a grouping of Genovesa mockingbirds with other *M. parvulus* populations. A fifth explanation, proposed by Hoeck et al. (2010), is incomplete lineage sorting (Nichols, 2001; Funk and Omland, 2003). Under this scenario, ancestral polymorphisms are present in the parental population prior to divergence.

It is not possible to distinguish between the five scenarios based on one genetic locus alone, and thus more unlinked genetic data are needed in addition to the maternally-inherited mtDNA sequences analysed by Arbogast et al. (2006). Hoeck et al. (2010) analysed variation at 16 microsatellite loci and found that Genovesa mockingbirds grouped with other *M. parvulus* populations, making scenarios of introgression or incomplete lineage sorting most likely. Another recent study analysed mitochondrial and nuclear loci of all Mimidae (Lovette et al., 2012) and confirmed known phylogenetic patterns among Galápagos mockingbirds, but its only *M. parvulus* samples were from Isabela and Santa Cruz, preventing closer analysis of many relationships among Galápagos mockingbirds. Here we determine which of these scenarios most likely explains the evolutionary history of Genovesa mockingbirds, using a combination of unlinked and differentially inherited genetic markers. We compare the gene trees of mitochondrial NADH dehydrogenase subunit 2 (*ND2*) and the cytochrome b (*CYTB*) genes with the autosomal beta-fibrinogen gene intron 7 (*FIB7*) and the transforming growth factor β2 intron (*TGF*) sequences, along with data on fragment length polymorphism of 26 microsatellite loci. In addition, we include morphometric data in this study to investigate if Genovesa mockingbirds are phenotypically distinct from their potential parental species. Thus, we use new, independent, and extensive samples from most Galápagos mockingbird populations to repeat the *ND2* analysis reported by Arbogast et al. (2006) and confirm their major findings. We provide entirely new data on *CYTB* and nuclear genetic variation. The combined data, together with morphological analyses, allow us to tackle the reasons for Genovesa mockingbird's enigmatic position in mtDNA gene trees (Arbogast et al., 2006; Štefka et al., 2011). Our genetic data are most compatible with the hypothesis (iv) of female-mediated introgression from San Cristóbal, making the Genovesa mockingbird a population of mixed ancestry.

2. Materials and methods

2.1. Samples

We (PEAH, LFK, PGP, PRG and BRG) captured the birds for this study with mist nets or potter traps, and took blood samples from individuals collected on 15 islands between 2004 and 2008 (Fig. 1a). Sample sizes from the different locations varied between mtDNA and nuclear sequencing analyses (101 individuals in total, comprising a subset of the individuals used for microsatellite analysis; Table 1), microsatellite analysis (543 samples from Hoeck et al. (2010), plus 4 additional ones from Santa Cruz; Table 2), and morphological analysis (801 individuals in total; Table 2). We collected blood samples on filter paper after a small puncture of the wing vein of live birds. We extracted genomic DNA using the QIAamp DNA Mini Kit (Qiagen; Hoeck et al., 2009) and standardised concentrations of DNA extracts at 20 ng/μL

Table 1
Sample sizes and diversity statistics, as well as GenBank accession numbers for the two mitochondrial genes (*ND2* = NADH dehydrogenase subunit 2; *CYTB* = Cytochrome b) and the two nuclear introns (following page; *FIB7* = Beta-fibrinogen gene; *TGF* = Transforming growth factor β2) analysed in Galápagos mockingbirds.

Island	Locus	*N*	#ht	hd ± SD	π(MPD) ± SD	π_n ± SD	GenBank
Champion	*ND2*	10	1	0 ± 0	0 ± 0	0 ± 0	KF411070
Española	*ND2*	6	2	0.533 ± 0.172	0.5333 ± 0.5077	0.00053 ± 0.00058	KF411074, 75
Fernandina	*ND2*	1	1	0 ± 0	0 ± 0	0 ± 0	KF411078
Gardner-by-Española	*ND2*	6	1	0 ± 0	0 ± 0	0 ± 0	KF411075
Gardner-by-Floreana	*ND2*	10	1	0 ± 0	0 ± 0	0 ± 0	KF411070
Genovesa	*ND2*	10	1	0 ± 0	0 ± 0	0 ± 0	KF411079
Isabela	*ND2*	6	4	0.800 ± 0.172	3.6667 ± 2.1551	0.00365 ± 0.00248	KF411078, 80–82
Marchena	*ND2*	6	2	0.333 ± 0.215	0.3333 ± 0.3801	0.00033 ± 0.00044	KF411083, 84
Pinta	*ND2*	5	2	0.600 ± 0.175	2.4000 ± 1.5567	0.00239 ± 0.00181	KF411085, 86
Rábida	*ND2*	2	2	1.000 ± 0.500	8.0000 ± 6.0000	0.00797 ± 0.00845	KF411083, 87
San Cristóbal	*ND2*	7	2	0.286 ± 0.196	0.2857 ± 0.3409	0.00029 ± 0.00039	KF411071, 72
Santa Cruz	*ND2*	7	1	0 ± 0	0 ± 0	0 ± 0	KF411073
Santa Fé	*ND2*	7	2	0.286 ± 0.196	0.2857 ± 0.3409	0.00029 ± 0.00039	KF411076, 77
Santiago	*ND2*	9	6	0.917 ± 0.073	2.3333 ± 1.4034	0.00232 ± 0.00159	KF411088-93
Wolf	*ND2*	3	1	0 ± 0	0 ± 0	0 ± 0	KF411078
Total	*ND2*	*95*	*24*	*0.918 ± 0.015*	*39.5306 ± 17.3232*	*0.03937 ± 0.01911*	*KF411070-93*
Champion	*CYTB*	7	1	0 ± 0	0 ± 0	0 ± 0	KF411094
Española	*CYTB*	6	1	0 ± 0	0 ± 0	0 ± 0	KF411096
Fernandina	*CYTB*	6	3	0.733 ± 0.155	0.8667 ± 0.7008	0.00152 ± 0.00142	KF411099-101
Gardner-by-Española	*CYTB*	5	1	0 ± 0	0 ± 0	0 ± 0	KF411096
Gardner-by-Floreana	*CYTB*	6	1	0 ± 0	0 ± 0	0 ± 0	KF411094
Genovesa	*CYTB*	5	1	0 ± 0	0 ± 0	0 ± 0	KF411097
Isabela	*CYTB*	5	2	0.600 ± 0.175	0.6000 ± 0.5622	0.00105 ± 0.00115	KF411102, 03
Marchena	*CYTB*	4	1	0 ± 0	0 ± 0	0 ± 0	KF411104
Pinta	*CYTB*	5	2	0.400 ± 0.237	0.4000 ± 0.4351	0.00070 ± 0.00089	KF411105, 06
Rábida	*CYTB*	5	2	0.600 ± 0.175	3.0000 ± 1.8741	0.00525 ± 0.00384	KF411107, 08
San Cristóbal	*CYTB*	5	1	0 ± 0	0 ± 0	0 ± 0	KF411095
Santa Fé	*CYTB*	7	1	0 ± 0	0 ± 0	0 ± 0	KF411098
Total	*CYTB*	*66*	*15*	*0.908 ± 0.017*	*19.3161 ± 8.6588*	*0.03383 ± 0.01681*	*KF411094-108*
Champion	*FIB7*	22	2	0.091 ± 0.081	0.0909 ± 0.1682	0.00011 ± 0.00023	KF411109, 14
Española	*FIB7*	16	2	0.325 ± 0.125	0.6500 ± 0.5305	0.00079 ± 0.00072	KF411113, 16
Fernandina	*FIB7*	4	1	0 ± 0	0 ± 0	0 ± 0	KF411112
Gardner-by-Española	*FIB7*	14	2	0.264 ± 0.136	0.5275 ± 0.4674	0.00064 ± 0.00064	KF411113, 16
Gardner-by-Floreana	*FIB7*	20	3	0.195 ± 0.115	0.3000 ± 0.3263	0.00036 ± 0.00044	KF411109, 18, 19
Genovesa	*FIB7*	16	2	0.325 ± 0.125	2.2750 ± 1.3174	0.00276 ± 0.00179	KF411112, 17
Isabela	*FIB7*	12	2	0.530 ± 0.076	0.5303 ± 0.4735	0.00064 ± 0.00065	KF411112, 21
Marchena	*FIB7*	12	1	0 ± 0	0 ± 0	0 ± 0	KF411112
Pinta	*FIB7*	10	1	0 ± 0	0 ± 0	0 ± 0	KF411112
Rábida	*FIB7*	4	3	0.833 ± 0.222	2.1667 ± 1.4988	0.00263 ± 0.00217	KF411110, 12, 21
San Cristóbal	*FIB7*	16	1	0 ± 0	0 ± 0	0 ± 0	KF411111
Santa Cruz	*FIB7*	14	2	0.143 ± 0.119	0.1429 ± 0.2187	0.00017 ± 0.00030	KF411112, 15
Santa Fé	*FIB7*	12	1	0 ± 0	0 ± 0	0 ± 0	KF411112
Santiago	*FIB7*	24	2	0.083 ± 0.075	0.0833 ± 0.1602	0.00010 ± 0.00022	KF411112, 20
Wolf	*FIB7*	6	1	0 ± 0	0 ± 0	0 ± 0	KF411112
Total	*FIB7*	*202*	*11*	*0.685 ± 0.027*	*3.0532 ± 1.5963*	*0.00370 ± 0.00214*	*KF411109-21*
Champion	*TGF*	6	1	0 ± 0	0 ± 0	0 ± 0	KF411123
Española	*TGF*	4	2	0.500 ± 0.265	0.5000 ± 0.5191	0.00090 ± 0.00112	KF411124, 25
Fernandina	*TGF*	4	2	0.667 ± 0.204	1.3333 ± 1.0249	0.00241 ± 0.00221	KF411128, 31
Gardner-by-Española	*TGF*	2	1	0 ± 0	0 ± 0	0 ± 0	KF411125
Gardner-by-Floreana	*TGF*	6	1	0 ± 0	0 ± 0	0 ± 0	KF411129
Genovesa	*TGF*	6	3	0.733 ± 0.155	1.1333 ± 0.8472	0.00205 ± 0.00177	KF411122, 27, 28
Isabela	*TGF*	8	3	0.714 ± 0.123	1.0000 ± 0.7481	0.00181 ± 0.00154	KF411122, 28, 31
Marchena	*TGF*	12	2	0.409 ± 0.133	0.4091 ± 0.4031	0.00074 ± 0.00082	KF411122, 28
Pinta	*TGF*	4	2	0.500 ± 0.265	0.5000 ± 0.5191	0.00090 ± 0.00112	KF411122, 28
Rábida	*TGF*	2	2	1.000 ± 0.500	2.0000 ± 1.7321	0.00361 ± 0.00442	KF411128, 31
San Cristóbal	*TGF*	14	1	0 ± 0	0 ± 0	0 ± 0	KF411126
Santa Cruz	*TGF*	10	3	0.511 ± 0.164	0.5556 ± 0.4943	0.00100 ± 0.00101	KF411122, 28, 30
Santa Fé	*TGF*	8	1	0 ± 0	0 ± 0	0 ± 0	KF411122
Santiago	*TGF*	6	2	0.600 ± 0.129	0.6000 ± 0.5477	0.00108 ± 0.00114	KF411122, 28
Wolf	*TGF*	6	2	0.533 ± 0.172	0.5333 ± 0.5077	0.00096 ± 0.00106	KF411122, 31
Total	*TGF*	*98*	*6*	*0.714 ± 0.026*	*1.0940 ± 0.7244*	*0.00198 ± 0.00145*	*KF411122-31*

N = number of haplotypes (i.e. the number of birds for mitochondrial data, or twice the number of birds for nuclear data); #ht = number of distinct haplotypes; hd ± SD = haplotype (gene) diversity ± standard deviation; π(MPD) ± SD = mean pairwise sequence difference ± standard deviation; π_n ± SD = nucleotide diversity ± standard deviation; GenBank = GenBank accession number(s) of sequences from each island; note that the following pairs or triplets of nuclear sequences are identical, but have different GenBank accession numbers because they occur in more than one species: KF411109 = KF411110, KF411116 = KF411117, KF411122 = KF411123, KF411125 = KF411126 = KF411127, KF411128 = KF411129.

Table 2
Sample sizes for microsatellite analysis and morphological measurements, and island abbreviations used in Fig. 3.

Island	Microsatellites	Morphology	Abbreviation
Champion	48	74	Cham
Española	87	182 (PGP: 94)	Esp
Fernandina	24		
Gardner-by-Española	10	10	G.Esp
Gardner-by-Floreana	69	118	G.Flo
Genovesa	37	103 (PGP: 103)	Geno
Isabela	62	88 (PGP: 56)	Isab
Marchena	38	39	March
Pinta	27		
Rábida	21	21	Ráb
San Cristóbal	37	37	S.Cris
Santa Cruz	39	82 (PGP: 20)	S.Cruz
Santa Fé	21	20	S.Fé
Santiago	27	27	Santi
Total	*547*	*801*	

PGP indicates the size of the subset of morphological samples measured by P.G. Parker's group. The other measurements are from the group of P.E.A. Hoeck and L.F. Keller.

(Quant-iT PicoGreen dsDNA Quantitation, Invitrogen). All samples are stored in freezers at the University of Zurich, Switzerland and still in use for ongoing studies.

2.2. Mitochondrial and nuclear gene analysis

2.2.1. PCR amplification and sequencing

Polymerase chain reactions (PCR) were conducted in 10 μL reaction volume and the following concentrations: 0.25 μM of each primer (Table 3), 0.1 U GoTaq polymerase (Promega), 0.2 mM of each dNTP, GoTaq reaction buffer, and 20 ng of template DNA. The PCR profile included 2 min preheating at 94 °C followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing (for annealing temperatures see Table 3) and 45 s extension at 72 °C and a final extension at 72 °C for 10 min. PCR was done in a GeneAmp PCR System 9700 or a Veriti 96 Well Thermal Cycler (both Applied Biosystems). We checked for PCR amplification on a 1% agarose gel and subsequently cleaned successful amplifications from non-incorporated primers by applying a standard ExoSAP protocol. Finally, we did forward and reverse sequencing on a 3730 DNA Analyser (Applied Biosystems) using BigDye Terminator (v3.1; Applied Biosystems) chemistry, Better Buffer (Web Scientific) and the primers from Table 3 for mitochondrial sequences or universal M13 primers (Schuelke, 2000) for nuclear sequences.

2.2.2. Sequence analysis

Base calling and visual inspection of trace files was done with Sequencing Analysis Software v5.1 (Applied Biosystems). We aligned mitochondrial and nuclear sequences manually in BioEdit (Hall, 1999) and trimmed sequences to constant length (Table 3). We used FinchTV v1.4.0 (Geospiza Research Team, http://www.geospiza.com/Products/finchtv.shtml) for visual control of polymorphic sites and ambiguous bases.

Both nuclear sequences *FIB7* and *TGF* revealed several heterozygous sites. Their phases were reconstructed using Phase v2.1 (Stephens et al., 2001; Stephens and Donnelly, 2003) in the DnaSP v5 package (Librado and Rozas, 2009). Phase reconstruction was unambiguous, with phase probabilities of at least 0.98 at all heterozygous sites. Finally, we detected identical sequences using CleanCollapse v1.0.5 (Ray, 2006). Arlequin v3.5 (Excoffier and Lischer, 2010) was used to calculate the diversity statistics shown in Table 1.

2.2.3. Phylogenetic reconstruction

One sample of each haplotype was used for phylogenetic analysis. We reconstructed two separate phylogenies for each of the two mitochondrial genes, with genes partitioned according to codon position. Stationarity of base frequencies among sequences was assessed with a χ^2-test using PAUP* v4.0b10 (Swofford, 2002). None of the partitions showed differing base frequencies among samples and examined genes (all $p > 0.9$), nor had any codon position reached substitution saturation as examined using the test by Xia et al. (I_{SS} was always significantly smaller than $I_{SS.C}$ for symmetrical and asymmetrical trees, $p < 0.00005$) (Xia et al., 2003; Xia, 2009), as implemented in the software DAMBE v5.2.9 (Xia and Xie, 2001). We applied MrModeltest v2.3 (Nylander, 2004) to determine the best model of nucleotide evolution based on the Akaike Information Criterion (Akaike, 1974) (selected models: *ND2* position 1: HKY + I, *ND2* position 2: HKY + I, *ND2* position 3: HKY + G, *CYTB* position 1: GTR + I, *CYTB* position 2: K80, *CYTB* position 3: HKY). MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) was used to generate a Metropolis coupled Markov chain Monte Carlo search and to determine Bayesian posterior probabilities under the best-fit model (partitioned by codon position) using *Mimus longicaudatus* as an outgroup for *ND2* (GenBank accession number EF468200; Lovette and Rubenstein, 2007) and *Toxostoma redivivum* as an outgroup for *CYTB* (GenBank accession number AY124543; Barhoum and Burns, 2002), which were close relatives of Galápagos mockingbirds for which sequences were available on GenBank (Lovette et al., 2012). Searches of 8,000,000 generations with 50% burn-in for *ND2* and 3,000,000 generations with 25% burn-in for *CYTB* with

Table 3
mtDNA and nuclear loci sequenced in this study. Their primer sequences, annealing temperature (T_A), analysed sequence length (in base pairs) and forward and reverse primer sequences are shown.

Locus	Primer sequence 5′-3′	T_A	Length
mtDNA: NADH dehydrogenase subunit 2 gene (*ND2*)	L5215[1]: TAT CGG GCC CAT ACC CCG AAA AT H1064[2]: CTT TGA AGG CCT TCG GTT TA	55	1004
mtDNA: Cytochrome b (*CYTB*)	L14996_FW2[3]: AAY ATY TCW GYH TGA TGA AAY TTY GG H16064[3]: CCT CAN TYT TTG GYT TAC AAG RCC	46[a]	571
Nuclear: Beta-fibrinogen gene intron 7 (*FIB7*)	FIB-BI7U[4]: tgt aaa acg acg gcc agt GGA GAA AAC AGG ACA ATG ACA ATT CAC FIB-BI7L[4]: cag gaa aca gct atg acc TCC CCA GTA GTA TCT GCC ATT AGG GTT	55	825
Nuclear: Transforming growth factor β2 intron (*TGF*)	TGFB2-5F[5]: tgt aaa acg acg gcc agt GAA GCG TGC TCT AGA TGC TG TGFB2-5F[5]: cag gaa aca gct atg acc AGG CAG CAA TTA TCC TGC AC	66	554

Universal primer sequences (Schuelke, 2000) added to the locus-specific sequences are printed in lower case letters.
Superscript numbers after primer names refer to these references: [1] J. Cracraft and K. Helm-Bychowski in Hackett (1996); [2] Drovetski et al. (2004); [3] Sorenson (2003); [4] Prychitko and Moore (1997); [5] Primmer et al. (2002).
T_A = annealing temperature in °C; Length = analysed sequence length in base pairs.
[a] We used the Qiagen Multiplex Master Mix, 0.25 μM of each primer, and 40 ng template DNA at cycling conditions according to manufacturer's instructions.

samples every 100 generations were performed. Runs converged later for *ND2* than *CYTB* and hence, number of generations and burn-in differ for the two genes to ensure that only converged runs were sampled, leading to effective sample sizes well above 200 for all parameters. Trees were visualised with FigTree v1.2 (Rambaut, 2009).

We performed molecular dating only for the longer and geographically more completely represented *ND2* sequence data. We applied a relaxed clock model with estimated uncorrelated lognormal rates in BEAST v1.5.4 (Drummond and Rambaut, 2007) using input files generated with BEAUti v1.5.4 (Drummond and Rambaut, 2007). We simulated a birth–death speciation process starting from a random tree and unlinked the HKY + G + I model parameters between codon positions. The gamma distribution was approximated with 6 categories. We applied a lognormal prior with a mean of 1,500,000 years and standard deviation (log transformed) of 0.5 on the height of the root (95% of values within 581,600–3,013,000 years). This prior covers the estimated ages of most islands currently above water in the Galápagos archipelago (Geist, 1996; D. Geist, 2005–2008, unpublished data; Poulakakis et al., 2012) and includes the likely age estimates from Arbogast et al. (2006). We did not impose any further constraints on other node ages. We performed four independent runs of 10,000,000 generations each and checked for convergence of runs in Tracer v1.5.0 (Rambaut and Drummond, 2009). The software LogCombiner v1.5.4 and TreeAnnotator v1.5.4 (both Drummond and Rambaut, 2007) were used to combine the parameter estimates and trees of the four runs after exclusion of 10% burn-in at the start of each run, leading to effective sample sizes well above 200 for all parameters.

As the nuclear introns *FIB7* and *TGF* showed low levels of polymorphism within the Galápagos mockingbirds and high levels of haplotype sharing among islands, we constructed median-joining networks (Bandelt et al., 1999) with Network v4.5 (http://www.fluxus-engineering.com/sharenet.htm) and Network Publisher v1.1.0.7 (http://www.fluxus-engineering.com/nwpub.htm). All characters were weighted equally and an epsilon value of zero was chosen. Epsilon specifies a tolerance up to which mutational distances among haplotypes are considered equal during the search for median vectors (Bandelt et al., 1999).

2.3. Microsatellite analysis

We used microsatellite genotypic data for 26 loci from Hoeck et al. (2010) and Hoeck and Keller (2012). To visualise patterns of variation in microsatellite repeat length, we conducted a factorial correspondence analysis using Genetix v4.05.2 (Belkhir, 2004). Plots including the first three axes were drawn in R v2.12.1 (R Development Core Team, 2010). Additionally, we analysed population structure using the admixture model with uncorrelated allele frequencies in Structure v2.3.3 (Pritchard et al., 2000). We sampled 600,000 repetitions after excluding the first 100,000 as burn-in. Runs were conducted for cluster numbers K ranging from 2 to 14 (i.e. the number of sampled island populations) and we determined the number of clusters best fitting the data based on ΔK (Evanno et al., 2005) using Structure Harvester (Earl and vonHoldt, 2012). Structure output plots were coloured and ordered in Distruct (Rosenberg, 2004).

2.4. Morphological analysis

We measured beak and tarsus lengths of all captured birds with digital calipers (to the nearest 0.1 mm), and wing length from the carpal joint to the tip of the unflattened longest primary feather to the nearest 1 mm using a ruler (according to Svensson, 1992). Most birds were measured by P.E.A. Hoeck and L.F. Keller. Measurements of Genovesa mockingbirds were only available from P.G. Parker's group (for sample sizes see Table 2). Due to differing techniques, measurements from the two groups differed systematically. We thus used morphological data of birds from Española, Santa Cruz, and Isabela Islands that were visited by both groups in order to correct for systematic measuring differences between observers. To this end, we performed linear regression analysis of the mean trait values per island (separately for mean wing, beak, and tarsus measurements) as measured by the Hoeck/Keller group against the corresponding measurements of the Parker group. Then, measurements of the Parker group were modified by multiplying them with the slope of the regression line (0.66 for beak, 0.93 for wing, and 0.64 for tarsus measurements, respectively) and adding the y-axis intercept (1.84 for beak, 1.06 for wing, and 10.49 for tarsus measurements, respectively). The high R^2 values (0.97 for beak, 0.97 for wing, and 0.85 for tarsus measurements, respectively) of the regression lines indicate that systematic differences in measurements can be efficiently corrected in this way. For further analyses we used Parker's corrected data and the raw measurements of the Hoeck/Keller group.

To test for morphological differences among island populations, we conducted a MANOVA on the standardised measurements (standardization through subtraction of mean and division with standard deviation), using the function "manova" in R. To get an idea of the similarities among island populations, we performed a cluster analysis of the mean beak, wing, and tarsus measurements per island, using Euclidean distances and the average distance among clusters, using the R function "hclust". To further investigate how genetic differences among island populations are related to morphological traits, and to conduct this analysis on uncorrelated linear combinations (correlations among standardised traits: $r_{\text{wing-beak}} = 0.73$, $r_{\text{wing-tarsus}} = 0.75$, $r_{\text{beak-tarsus}} = 0.54$), we performed a principal components analysis of wing, beak, and tarsus length using the R function "prcomp". We tested the three principal components for differences between islands with ANOVA. We determined significant pairwise comparisons using Tukey's honest significant differences in R.

3. Results

3.1. Mitochondrial and nuclear DNA sequences

We sequenced four loci to investigate the genetic diversity of Galápagos mockingbirds. The two mitochondrial genes revealed high nucleotide diversity over all islands ($\pi_n = 0.04$ for *ND2*; $\pi_n = 0.03$ for *CYTB*) in comparison to the two nuclear introns ($\pi_n = 0.004$ for *FIB7*; $\pi_n = 0.002$ for *TGF*; Table 1). The birds from Genovesa ranked among the most variable at the nuclear sequences (Table 1), although they only showed a single mitochondrial haplotype.

In order to investigate the genetic relatedness among Galápagos mockingbirds, we constructed phylogenetic trees of the mitochondrial sequences and median-joining networks of the nuclear introns. Furthermore, we estimated node ages for mitochondrial *ND2*. The trees derived from *CYTB* and *ND2* were topologically consistent; we therefore only show the tree based on the more complete and variable *ND2* dataset (Fig. 2). We found four well differentiated and statistically supported clades. The oldest split within Galápagos mockingbirds occurred ca. 500,000 (95% credible interval: 145,957–1,388,173) years ago and separated the mitochondrial haplotypes of birds from San Cristóbal, Genovesa, and Española together with Gardner-by-Española from all other islands (node 1 in Fig. 2). Mockingbirds on the former islands are considered to belong to three distinct species (*Mimus melanotis* on San Cristóbal, *M. macdonaldi* on Española, and *M. parvulus bauri* on Genovesa; Swarth, 1931; Remsen et al., 2012), but their mitochon-

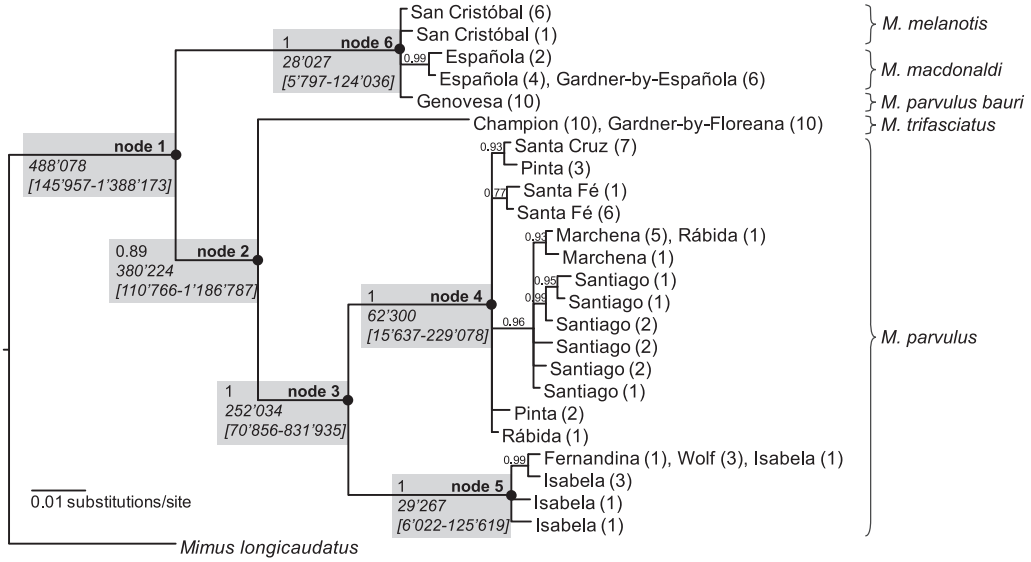

Fig. 2. Mitochondrial phylogeny. Phylogenetic tree derived from mitochondrial *ND2* sequences (consistent with phylogeny derived from a smaller dataset of *CYTB* sequences; data not shown). Shown above branches are Bayesian posterior probabilities and below branches in italics the mode and 95% highest posterior density intervals of split date estimates as derived from a relaxed clock model. Numbers in brackets behind island names refer to the number of birds with the respective haplotype. Note that we detected only one haplotype in birds from Genovesa which clustered with haplotypes of birds from San Cristóbal and Española.

drial haplotypes only split around 30,000 (5797–124,036) years ago (node 6). A second clade was formed by the 380,000 (110,766–1,186,787) year old split (node 2) of the Floreana mockingbird (*M. trifasciatus*) from all *M. parvulus* populations except the one on Genovesa Island. Another deep split (node 3) occurred around 250,000 (70,856–831,935) years ago within *M. parvulus*, separating the mitochondria of the birds on the western islands of Isabela, Fernandina, and Wolf, from the mitochondria of the birds on the central and northern islands of Santa Cruz, Santiago, Marchena, Pinta, Santa Fé, and Rábida. Note that the modal estimate of the age of this intraspecific split (node 3 in Fig. 2) was 6.3 times older (calculated from the MCMC samples; 95% credible interval: 2.6–17.2 times older) than the age of the separation of the mitochondrial haplotypes of the three species *M. melanotis* on San Cristóbal, *M. macdonaldi* on Española, and *M. parvulus bauri* on Genovesa (node 6). The age of the first split within Galápagos mockingbirds (node 1) was 11.9 (4.8–29) times older than the age of the most recent split at node 6.

Median-joining networks based on both nuclear introns, *FIB7* (Fig. 1b) and *TGF* (Fig. 1c), showed much less resolution than the mitochondrial sequences due to their lower variation (Table 1). However, they did not contradict the mitochondrial phylogenies, except for the placement of Genovesa mockingbirds: The birds from Genovesa showed two and three divergent haplotypes for *FIB7* and *TGF*, respectively. One of the *FIB7* haplotypes from Genovesa mockingbirds was shared with birds on Española Island and one mutational step away from birds on San Cristóbal Island (Fig. 1b). Similarly, one of the *TGF* haplotypes from Genovesa mockingbirds was shared with birds of Española and San Cristóbal Islands (Fig. 1c). This pattern was consistent with the mitochondrial data, where haplotypes of birds from these three islands grouped in the same clade, with 1–2 substitutions in *ND2* between birds from Genovesa and San Cristóbal Islands and 5–6 substitutions between birds from the latter two islands and Española Island. Contrary to the mitochondrial data, the second *FIB7* haplotype from Genovesa mockingbirds was shared with *M. parvulus* (Fig. 1b). Similarly, the second and third *TGF* haplotypes were shared with *M. parvulus* and *M. trifasciatus* (Fig. 1c). These mitochondrial and nuclear sequence data indicate a mixed genetic composition of Genovesa mockingbirds.

3.2. Autosomal microsatellite loci

To obtain a more representative picture of the autosomal genome, we investigated variation at additional nuclear loci: 26 autosomal microsatellites. A factorial correspondence analysis (Fig. 1d) revealed four groups that correspond to the four currently recognised species (Swarth, 1931; Remsen et al., 2012). The genotypes of Genovesa mockingbirds grouped with the other *M. parvulus* on axes 1 and 2. On the third axis, genotypes of Genovesa mockingbirds were more divergent, but still grouped most closely with the other *M. parvulus* (Fig. 1d). Hence, the microsatellite data showed that at these 26 autosomal markers, Genovesa mockingbirds are similar to the other *M. parvulus* populations. This finding is consistent with the Structure analysis for the best-fitting number of clusters (i.e. $K = 4$, Supplementary Fig. S1), where Genovesa mockingbirds also clustered with most *M. parvulus* populations (Supplementary Fig. S2). Contrary to the results of the factorial correspondence analysis (Fig. 1d), the Structure analysis for $K = 4$ grouped *M. melanotis* (San Cristóbal) and *M. macdonaldi* (Española and Gardner-by-Española) in the same cluster, while the *M. parvulus* populations on Isabela and Fernandina formed an own cluster (Supplementary Fig. S2).

3.3. Morphological analysis

Measurements of wing, beak, and tarsus length differed among island populations for each trait separately (ANOVA; wing, $F_{11,789} = 81.9$, $p < 0.001$; beak, $F_{11,789} = 214.0$, $p < 0.001$; tarsus, $F_{11,789} = 100.4$, $p < 0.001$), as well as combined (MANOVA, $F_{33,2367} = 71.0$, $p < 0.001$). In the cluster analysis (Fig. 3), Española mockingbirds (*M. macdonaldi*) and Floreana mockingbirds (*M. trifasciatus*) grouped separately from the other populations. San Cristóbal mockingbirds (*M. melanotis*) grouped together with the birds from Santiago among *M. parvulus* populations. Interestingly, Genovesa mockingbirds (*M. parvulus bauri*) did not cluster closely with any other population. We also conducted a principal component analysis of morphological measurements (Fig. 4, Supplementary Figs. S3 and S4). Principal component 1 (representing size differences, as all variables load in the same direction) explained 78% of the variation and separated *M. macdonaldi* (Española and

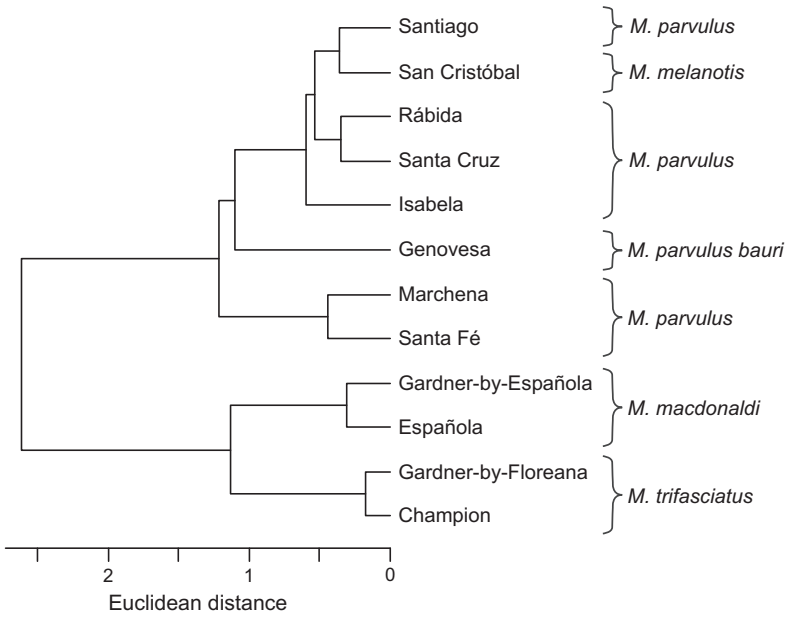

Fig. 3. Morphological dendrogram. Dendrogram constructed with Euclidean distances of mean morphological measurements (wing, beak, and tarsus length) per island population and average distances among clusters.

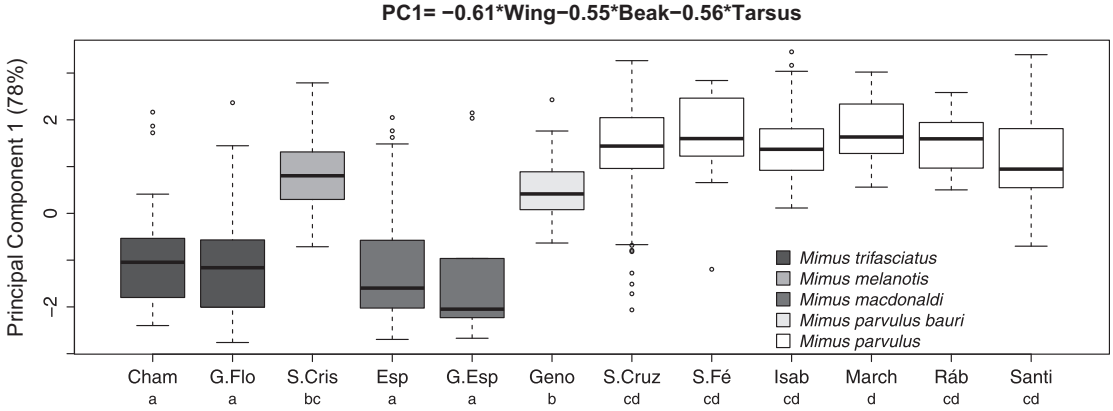

Fig. 4. Morphological measurements. Boxplots showing the first principal component of morphological measurements, representing size differences. Note that larger values represent smaller birds. The letters below the abbreviated island names (see Table 2 for translation) represent Tukey's honest significant differences; pairs of islands that are labelled with at least one identical letter do not differ significantly in their respective principle component; those that do not share at least one letter are significantly different. 78% of the variation is explained by this principal component. For principal components 2 and 3 see Supplementary Figs. S3 and S4.

Gardner-by-Española) and *M. trifasciatus* (Champion and Gardner-by-Floreana) from all other populations. The mean principal component 1 of the birds from Genovesa Island differed significantly from all populations of *M. parvulus*, which were not significantly different among themselves. The birds from San Cristóbal were on average intermediate between, but not significantly different from, the birds from Genovesa Island and most other *M. parvulus*. Taken together, wing, beak, and tarsus measurements separated *M. trifasciatus* and *M. macdonaldi* from the other Galápagos mockingbirds and, to a lesser extent, from each other. Among the remaining taxa, the Genovesa mockingbirds were more clearly different from other *M. parvulus* populations than *M. melanotis* from *M. parvulus*.

4. Discussion

We investigated the ancestry of Genovesa mockingbirds using a combination of sequence data from two mitochondrial and two nuclear genes, length polymorphisms at 26 autosomal microsatellite loci, and morphological measurements. These data show that Genovesa mockingbirds (*M. parvulus bauri*) are of mixed ancestry with mitochondria from *M. melanotis* and a nuclear genome largely, but not entirely, from *M. parvulus*. They are also morphologically distinct.

4.1. Genetic data reveal ancient introgression

Phylogenies derived from the mitochondrial genes *ND2* and *CYTB* revealed two important patterns. (a) The mitochondria of the mockingbirds from Genovesa (*M. parvulus bauri*), San Cristóbal (*M. melanotis*), and Española (*M. macdonaldi*) share a recent common ancestor, with mitochondria of the mockingbirds from Genovesa being most similar to those of birds from San Cristóbal. Two previous studies have described this surprising grouping of mitochondria from three species (Arbogast et al., 2006; Štefka et al., 2011). It shows that the mockingbirds on these three islands must have exchanged genes relatively recently, until between 124,036 and 5797 years ago (modal estimate of 28,027 years ago; node 6

in Fig. 2). Otherwise they could not have shared a common ancestor at that time (but gene flow may have persisted until more recently; Nichols, 2001). (b) The mitochondrial haplotypes of the birds on Genovesa, San Cristóbal, and Española separated from those of other *M. parvulus* populations very long ago (145,957–1,388,173 years ago with a modal estimate of nearly 500,000 years ago; node 1 in Fig. 2). This split (node 1 in Fig. 2) occurred 4.8–29 times (modal estimate of 11.9 times) earlier than the split among the mtDNA of the three species on Genovesa, San Cristóbal, and Española. In this study we investigated if the close clustering of Genovesa mockingbirds with other species and the long separation of Genovesa mockingbirds from conspecific populations was a peculiarity of the mitochondrial DNA. To that end we analysed the sequences of two nuclear introns and the length polymorphisms at 26 microsatellite loci. We detected two divergent haplotypes for *FIB7* and three divergent haplotypes for *TGF* in birds from Genovesa (*M. parvulus bauri*). One haplotype of each locus grouped with haplotypes of *M. melanotis* (San Cristóbal) and *M. macdonaldi* (Española and Gardner-by-Española), while the other haplotypes grouped with *M. parvulus* haplotypes (Fig. 1b and c). Autosomal microsatellite data, on the other hand, grouped the mockingbirds on Genovesa together with other *M. parvulus* populations, while the remaining three species (*M. melanotis*, *M. macdonaldi*, and *M. trifasciatus*) formed three distinct groups in the factorial correspondence analysis (Fig. 1d), or two clusters in the Structure analysis (Supplementary Fig. S2). This result is concordant with a previous analysis of our samples at 16 microsatellite loci (Hoeck et al., 2010), with the exception that the ten additional loci included here allowed to separate *M. melanotis* from *M. macdonaldi* in the factorial correspondence analysis, but not in the Structure analysis. To sum up, the microsatellites and the majority, but not all, of the nuclear haplotypes group Genovesa mockingbirds with other *M. parvulus* populations.

This genetic pattern provides a resolution of the evolutionary history of Genovesa mockingbirds. In line with Hoeck et al. (2010), two of the potential explanations of Arbogast et al. (2006) for the unexpected mitochondrial clustering of Genovesa mockingbirds with *M. melanotis* and *M. macdonaldi* can be rejected: (i) plumage convergence of Genovesa mockingbirds with *M. parvulus*, and (ii) plumage symplesiomorphy of Genovesa mockingbirds and *M. parvulus*. Both scenarios would require that all genetic loci reveal the same clades (except the markers linked to loci coding for plumage characteristics). Scenario (iii), which implies differential introgression of plumage-determining genes from *M. parvulus* into the Genovesa mockingbird population (Arbogast et al., 2006), is also unlikely because the majority of the analysed autosomal genetic variation, and not just a few loci, group Genovesa mockingbirds clearly with *M. parvulus*. It is conceivable, albeit unlikely, that strong gene flow from *M. parvulus* into the Genovesa mockingbird population might have replaced most of the nuclear genome of Genovesa mockingbirds, but not their mitochondrial genome. Such a scenario would require considerable introgression at many unlinked autosomal loci, but none at the mitochondrion, and thus does not appear to be parsimonious. Similar effects could come from an invasion of *M. parvulus* onto Genovesa Island, followed by replacement of a resident *M. melanotis* population. Currat et al. (2008) showed that in such cases of range expansion, introgression is most likely to occur into the invading species and preferentially involves loci like mtDNA that are subject to reduced gene flow (Birky et al., 1989). Such a scenario of species replacement with introgression is similar to its reverse scenario (iv) (Arbogast et al., 2006), female-mediated introgression of mitochondrial and some nuclear genes from *M. melanotis* into Genovesa mockingbirds.

This scenario (iv) would involve the following events: Genovesa Island was colonised by a population of birds originating from a *M. parvulus* population. Between 124,036 and 5797 years ago (modal estimate of 28,027 years ago; node 6 in Fig. 2) at least one female (or more, and possibly, but not necessarily, also some males) from San Cristóbal dispersed to Genovesa. The immigrant female(s) introduced their mitochondrial haplotypes into the population on Genovesa, where the haplotype observed today eventually became fixed. In addition to their mitochondrial haplotypes, these birds also introduced distinct nuclear haplotypes into the population on Genovesa, but none of them reached fixation at the examined loci. Mitochondrial haplotypes are more strongly influenced by genetic drift than autosomal genes due to their four times smaller effective population size, and are therefore expected to reach fixation faster than autosomal polymorphisms (Takahata and Slatkin, 1984; Funk and Omland, 2003). The scenario outlined above is consistent with our microsatellite data, which show a close relationship between the birds from Genovesa and other *M. parvulus* populations. The most parsimonious explanation for this pattern is that it was the maternally inherited mitochondrial haplotype that introgressed into the population and replaced the resident haplotype.

Introgression of genes transmitted by females may generally be more likely, as females are usually the dispersing sex in birds (Greenwood and Harvey, 1982), including Genovesa mockingbirds (Curry and Grant, 1989). However, it is unknown if these patterns of female-biased dispersal within islands (Curry and Grant, 1989) also hold for long-distance dispersal between islands. Chances for the complete replacement of *M. parvulus* mtDNA with mtDNA from *M. melanotis* would be much increased by population bottlenecks. Interestingly, declines in genetic diversity of the grey warbler-finch (*Certhidea fusca*) population on Genovesa may be explained by periodic cycles of population increases during wet El Niño events followed by bottlenecks during arid times (Farrington and Petren, 2011). Similarly, the sharp-beaked ground-finch (*Geospiza difficilis*) population on Genovesa seems to have undergone a genetic bottleneck which aided the introgression of small ground-finch (*G. fuliginosa*) genes (Grant and Grant, 2008b). Thus, it is plausible that bottlenecks also affected the mockingbirds on Genovesa. Additionally, it is possible that selection on mtDNA may have contributed to the spread of the introgressed mitochondrial lineage. Although controversial (Karl et al., 2012), selection on mtDNA has been suggested in various taxa (Bazin et al., 2006); (but see Berry, 2006; Mulligan et al., 2006; Albu et al., 2008; Nabholz et al., 2008) including parasitic wasps (genus *Nasonia*; Oliveira et al., 2008), killer whales (*Orcinus orca*; Foote et al., 2011), and humans (Mishmar et al., 2003), as well as in simulation studies (Bonnet, 2012).

An alternative explanation to introgression for the observed genetic patterns can be (v) incomplete lineage sorting (Nichols, 2001; Funk and Omland, 2003; Hoeck et al., 2010). However, this explanation seems very unlikely in our case. For incomplete lineage sorting to explain our results, at least all populations of *M. parvulus*, *M. macdonaldi*, and *M. melanotis* would have had to exchange genes during almost 145,957–1,388,173 years (modal estimate of approximately 460,000 years; from node 1 until node 6 in Fig. 2). Starting between 124,036 and 5797 years ago (modal estimate of 28,027 years ago; node 6 in Fig. 2), exchange of mitochondrial genes among species would have stopped and complete lineage sorting according to species affiliation would have occurred on all islands except on Genovesa. Hence, lineage sorting would have had to occur in a period of time (since node 6 in Fig. 2) that was 11.9 (4.8–29) times shorter than the long period of time (from node 1 to node 6 in Fig. 2) during which all major lineages must have been segregating without lineage sorting. Although some of the central islands were fused during parts of the Pleistocene, this was never the case for all islands inhabited now by *M. parvulus*, *M. macdonaldi*, and *M. melanotis* (Geist, 1996; D. Geist, 2005–2008, unpublished data; Poulakakis et al., 2012). Thus, it is difficult to imagine such a drastic change in the biology of Galápagos mock-

ingbirds that would have changed their dispersal behaviour to cause a shift from a population genetically mixing across most of the archipelago to many well-differentiated island populations, as found today (Hoeck et al., 2010). Furthermore, it is unlikely that the random process of lineage sorting would occur exactly according to species affiliation in all cases except in Genovesa mockingbirds. Hence, incomplete lineage sorting is extremely unlikely to account for the observed genetic pattern in Galápagos mockingbirds.

4.2. Colonisation history of Galápagos mockingbirds

Our mitochondrial phylogeny is consistent with a previously published mitochondrial phylogeny (Arbogast et al., 2006). A later study investigated the phylogenies of Galápagos mockingbirds and two of their parasites (Štefka et al., 2011). Their mitochondrial phylogenies of the host and both parasites were largely consistent with our phylogeny, with one exception. Štefka et al. (2011) found that the Floreana mockingbird was nested within *M. parvulus*, making the latter species paraphyletic. However, this paraphyly had low support and was not recovered by either our analysis or by Arbogast et al. (2006) or Lovette et al. (2012).

The order of splits in the presented phylogeny can be used to speculate about the colonisation history of mockingbirds in the Galápagos archipelago (Supplementary Fig. S5), as has similarly been done by Arbogast et al. (2006). *M. melanotis* from San Cristóbal and *M. macdonaldi* from Española formed the basal lineage among Galápagos mockingbirds and fittingly inhabit the two oldest currently exposed islands (Geist, 1996; D. Geist, 2005–2008, unpublished data), suggesting that the first mockingbirds on the archipelago arrived on San Cristóbal or Española. The next split (node 2 in Fig. 2) separated the Floreana mockingbirds (*M. trifasciatus*) from *M. parvulus*, making it likely that Floreana was the third island to be colonised. From there, mockingbirds would have invaded the central islands, probably including Genovesa. Depending on the exact timing of this split, some of the central islands were then connected by land (Poulakakis et al., 2012). Later on, mockingbirds from the central islands crossed to the young island of Isabela (node 3 in Fig. 2), from where soon afterwards populations on Wolf and on the youngest island of the archipelago, Fernandina, were established. Roughly at the same time, introgression from San Cristóbal into the population on Genovesa occurred. This colonisation history matches well with the order of emergence of the Galápagos islands (Geist, 1996; D. Geist, 2005–2008, unpublished data; Poulakakis et al., 2012) and follows the prevailing pattern of mainly southerly, southeasterly, and easterly winds (Jackson, 1991; Arbogast et al., 2006).

The Galápagos archipelago is inhabited by one of the best-known adaptive radiations, the Darwin's finches (Grant and Grant, 2008b). It is intriguing why one group, the Darwin's finches, radiated extensively with multiple species occurring in sympatry, while in another group on the same archipelago, the Galápagos mockingbirds, only four species occur and none of them live in sympatry with another mockingbird species (Grant and Grant, 2008b). Possible explanations for this observation include different times since colonisation of the islands or since the start of radiation. Although the age estimates are not very precise, our data estimate the first split within Galápagos mockingbirds (not the colonisation of the archipelago) to have occurred between 145,957–1,388,173 years ago (modal estimate of 488,078 years ago) years ago. This estimate is more recent than the current estimate (obtained with different methods than our estimates) of 1,650,000 years for the first split within Darwin's finches (Petren et al., 2005). Thus, the more recent start of the Galápagos mockingbird radiation may in part explain their lower diversity. However, their generalist feeding behaviour may be a more important explanation (Arbogast et al., 2006; Grant and Grant, 2008b, chapter 11).

4.3. Morphological analysis

Our analysis of morphological measurements showed that the introgressed population on Genovesa is more distinct from *M. parvulus*, than the birds from San Cristóbal (*M. melanotis*) are from many *M. parvulus* populations (Figs. 3 and 4). In fact, *M. melanotis* clustered with *M. parvulus* from Santiago (Fig. 3). It must have been this morphological similarity that led both Charles Darwin and John Gould to consider the population on Santiago to be the same species as that on San Cristóbal (Gould, 1837, 1841; Darwin, 1839). All *M. parvulus* populations (including Genovesa mockingbirds) and *M. melanotis* are more similar to each other than to any of the other, morphologically distinct populations of the two species *M. trifasciatus* and *M. macdonaldi*. Abbott and Abbott (1978) performed a canonical variates analysis of beak, tarsus, and wing measurements. Similar to our results, they found that *M. trifasciatus* and *M. macdonaldi* are morphologically clearly distinct from each other and from all other Galápagos mockingbirds. *M. melanotis* was morphologically similar to some *M. parvulus* populations, and Genovesa mockingbirds were at the edge of the *M. parvulus* cluster. Abbott and Abbott (1978) also calculated for each population a measure of morphological dissimilarity towards all other Galápagos mockingbird populations. This dissimilarity measure was higher for the birds from Genovesa than for those from San Cristóbal, but several other populations of *M. parvulus* also showed high dissimilarity measures (Abbott and Abbott, 1978). Taken together, the results of our morphological analysis agreed with that of Abbott and Abbott (1978) and showed that the birds on Genovesa are not phenotypically intermediate between their suggested parental taxa *M. parvulus* and *M. melanotis*. However, introgression of *M. melanotis* into *M. parvulus bauri* may have nonetheless contributed to the phenotypic distinctiveness of Genovesa mockingbirds. In addition to gene flow, environmental conditions may be shaping mockingbird morphology, leading to small and short-beaked birds on low and arid islands and large and long-beaked birds on the more humid, higher and more diverse islands (Gould, 1837, 1841; Swarth, 1931; Bowman and Carter, 1971; Abbott and Abbott, 1978; Curry, 1989; Curry and Grant, 1990). Thus, convergent evolution may be part of the reason why mockingbirds on low and arid Genovesa are morphologically different from populations of *M. parvulus* on higher and larger islands, approaching, but not reaching, the beak and body size of mockingbirds on arid Española or Champion and Gardner-by-Floreana. Similarly, San Cristóbal mockingbirds may be morphologically similar to many *M. parvulus* populations because they also live on a higher, more humid and more diverse island.

4.4. Conclusions

Generally in line with previous studies (Arbogast et al., 2006; Hoeck et al., 2010), we showed that at nuclear loci the mockingbirds on Genovesa Island (*M. parvulus bauri*) are largely similar to other *M. parvulus* populations, but contain mitochondria and some autosomal loci that group them with *M. melanotis* and *M. macdonaldi*. The most parsimonious explanation for this genetic pattern is introgressive hybridization. The direction of hybridization cannot be unequivocally resolved, but it is much more likely that introgression from San Cristóbal into an *M. parvulus* population on Genovesa gave rise to this genetic pattern, because this direction of introgression only implies the replacement of one locus with relatively small effective population size, the mitochondrial DNA. In any case, the genetic composition of Genovesa mockingbirds is the result of ancient hybridization. Because hybridization occurred

among non-sister species, it led to a change in topology of the mitochondrial gene tree, reinforcing the importance of analysing multiple loci when reconstructing a phylogeny (Rheindt and Edwards, 2011).

The morphology of Genovesa mockingbirds is distinct, albeit not exactly intermediate between their parent species. The distinctiveness of Genovesa mockingbirds has also been recognised by systematics. The first description of the Genovesa mockingbird considered it a new species (*Nesomimus bauri*; Ridgway, 1894), but nowadays it is treated as subspecies *M. parvulus bauri* (Cody, 2005; Clements et al., 2012). Irrespective of its taxonomic label, the Genovesa mockingbird is a lineage of mixed genetic ancestry, but it remains difficult to judge to what extent the here described ancient introgression contributed to the morphological distinctiveness of Genovesa mockingbirds. If morphological distinctiveness and reproductive isolation of Genovesa mockingbirds from both parental species was increased by hybridization, this case could be considered one of incipient homoploid hybrid speciation. Continuing divergence and speciation of Genovesa mockingbirds may be facilitated by their geographic isolation from both parental species (Buerkle et al., 2000; Duenez-Guzman et al., 2009), but their current degree of reproductive isolation remains to be studied.

Data accessibility

DNA sequences are deposited on GenBank under accession numbers KF411070 to KF411131 (see Table 1).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.07.020.

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